FORM PTO-1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 620-179
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S APPLICATION NO. (If known, see 37 C.F.R. 1.5) 10/018826 (To Be Assigned)
INTERNATIONAL APPLICATION NO. **PCT/GB00/02449**	INTERNATIONAL FILING DATE **26 June 2000**	PRIORITY DATE CLAIMED **24 June 1999**

TITLE OF INVENTION

CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

APPLICANT(S) FOR DO/EO/US

DAVIS, Peter D.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information. PTO-1449 Form

JC03 Rec'd PCT/PTO 21 DEC 2001

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018826 (To Be Assigned)	PCT/GB00/02449	620-179

21. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):	
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO $740.00	
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00	
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	19	-20 =	0	X	$18.00	$ 0.00
Independent Claims	1	-3 =	0	X	$84.00	0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$280.00	$ 280.00

TOTAL OF ABOVE CALCULATIONS =	$ **1300.00**
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	0.00
SUBTOTAL =	$ **1300.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +	0.00
TOTAL NATIONAL FEE =	$ **1300.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +	$ 0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1280.00 – Small Entity = $640.00)	$ 0.00
TOTAL FEES ENCLOSED =	$ **1300.00**
	Amount to be: refunded $
	Charged $

a. ☒ A check in the amount of $1300.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Reg No 32955

SEND ALL CORRESPONDENCE TO:

Mary J. Wilson
SIGNATURE

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000

(for)

B. J. Sadoff
NAME

36,663 REGISTRATION NUMBER

December 21, 2001 Date

587547

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

DAVIS, Peter D. Atty. Ref.: **620-179**

Serial No. **10/018,826** Group:

Filed: **December 21, 2001** Examiner:

For: **CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS**

* * * * * * * * * * *

April 22, 2002

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

AMENDMENT

Responsive to the Notification dated February 21, 2002, entry and consideration of the following amendments and remarks are requested.

IN THE SPECIFICATION

Amend the specification as follows.

Page 12, delete the paragraph spanning lines 22 and 23 and insert the following therefor:

--KYITTIAGVM TLSQVKGFVR KNGVNEAKID EIKNDNVQDT AEQKVQLLRN WHQLHGKKEA YDTLIKDLKK ANLCTLAEKI QTII (SEQ ID NO:1)--.

Page 13, delete the paragraph spanning lines 1 and 2 and insert the following therefor:

--PATLY AVVENVPPLR WKEFVRRLGL SDHEIDRLEL QNGRCLREAQ YSMLATWRRR TPRREATLEL LGRVLRDMDL LGCLEDIEEA L. (SEQ ID NO:2)--

Page 27, delete the paragraphs spanning lines 18-21 and insert the following therefor:

--Figures 2A and 2B show the nucleic acid (SEQ ID NO:3) and predicted amino acid sequence (SEQ ID NO:4) of $C44H^{EXTRA}$—$FAS^{TM/CYTO}$

Figures 3A and 3B show the nucleic acid (SEQ ID NO:5) and predicted amino acid sequence (SEQ ID NO:6) of $C44H^{EXTRA/TM}FAS^{CYTO}$--

Page 28, delete the paragraphs spanning lines 16-19 and insert the following therefor:

--Figures 10A to 10D show the nucleic acid (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8) of Flt-$1^{EXTRA}FAS^{TM/CYTO}$.

Figures 11A to 11D show the nucleic acid (SEQ ID NO:9) and amino acid sequence (SEQ ID NO:10) of Flt-$1^{EXTRA/TM}Fas^{CYTO}$.--

Page 30, delete the paragraphs spanning lines 20-22 and insert the following therefor:

--5' primer 5' GCG**GAATTC**AGGGGCGGGCACTGGCAC 3' (SEQ ID NO:11)
EcoR1

3' primer 5' GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3' (SEQ ID NO:12)
XhoI--.

Page 31, delete the paragraphs spanning lines 1-9 and insert the following therefor:

--5' primer 5' AACG**TGATCA**TCCTTTGTCTTCTTCTTTTG 3' (SEQ ID NO:13)
BclI

3' primer 5' GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3' (SEQ ID NO:12)

XhoI

Fas^{CYTO}

5' primer 5' G**CCCGGG**GTGAAGAGAAAGGAAGTACAG 3 (SEQ ID NO:14)

SmaI

3' primer 5' GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3' (SEQ ID NO:12)

XhoI--.

Page 40, delete the paragraph on line 22 and insert the following therefor:

--5' primer 5' GC**GGGTAC**CGCGGCCAGCGGGCCTGGCGCC 3' (SEQ ID NO:15)--.

Page 41, delete the paragraph on line 1 and insert the following therefor:

--3' primer 5' GGC**GGATCC**GTCCGAGGTTCCTTGAACAGTGAGG 3' (SEQ ID NO:16)--.

Page 41, delete the paragraph on line 4 and insert the following therefor:

--5' primer 5' GCG**GGTACC**GCCGCCGGTCGGCGCCCGGGC 3' (SEQ ID NO:17)--.

Page 41, delete the paragraph on line 6 and insert the following therefor:

--3' primer 5' GGC**GGATCC**CTTTTCCTGGGCACCTTCTATTATG 3' (SEQ ID NO:18)--.

Page 43, delete the paragraphs spanning lines 23 and 24 and insert the following therefor:

--5' primer 5' GAGACCCTGGTGGACATCTTCCAGGAGTACCC 3' (SEQ ID NO:19)

3' primer 5' GGCTCCTTCCTCCTGCCCGGCTCACCGCCTCG 3' (SEQ ID NO:20)--.

Page 44, delete the paragraphs spanning lines 1 and 2 and insert the following therefor:

--5' primer 5' GAGCGGGAAATCGTGCGTGACATT 3' (SEQ ID NO:21)

3' primer 5' GATGGAGTTGAAGGTAGTTTCGTG 3' (SEQ ID NO:22)--.

Insert the attached paper copy of the Sequence Listing after the claims.

IN THE CLAIMS

Amend the claims as follows.

9. (Amended) A nucleic acid for use according to any one of the preceding claims encoding an amino acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D (SEQ ID NOs:4, 6, 8, or 10).

10. (Amended) A nucleic acid for use according to claim 9 having a nucleic acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D (SEQ ID NOs:3, 5, 7 or 9).

REMARKS

Reconsideration is requested.

The specification and claims have been amended above to include the attached paper copy of the Sequence Listing and sequence identifiers, in response to the attached Notification dated February 21, 2002.

The attached paper and computer-readable copies of the Sequence Listing are the same. No new matter has been added. A separate Statement to this effect is attached.

Attached is an executed Declaration, as required by the attached Notification.

The Examiner is requested to examine the claims attached, as annexes and marked Amended Sheet (pages 51-53), claims 1-17, attached to the International Preliminary Examination Report. A copy of the annexes should have been forwarded by the International Bureau however the Office is requested to advise the undersigned if a further copy of the same is required.

The attached and above are believed to be completely responsive to the Notification dated February 21, 2002, however the Office is requested to contact the undersigned if anything further is required in this regard and provide further time, without penalty of extension fee payments, for any further response which is deemed required.

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. **36,663**

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MARKED UP PAGES OF SPECIFICATION AND CLAIMS

Page 12, delete the paragraph spanning lines 22 and 23 and insert the following therefor:

--KYITTIAGVM TLSQVKGFVR KNGVNEAKID EIKNDNVQDT AEQKVQLLRN WHQLHGKKEA YDTLIKDLKK ANLCTLAEKI QTII (SEQ ID NO:1)--.

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--PATLY AVVENVPPLR WKEFVRRLGL SDHEIDRLEL QNGRCLREAQ YSMLATWRRR TPRREATLEL LGRVLRDMDL LGCLEDIEEA L. (SEQ ID NO:2)--

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Page 28, delete the paragraphs spanning lines 16-19 and insert the following therefor:

--Figures 10A to 10D show the nucleic acid (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8) of Flt-1^{EXTRA}FAS$^{TM/CYTO}$.

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EcoR1

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BclI

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Page 44, delete the paragraphs spanning lines 1 and 2 and insert the following therefor:

--5' primer 5' GAGCGGGAAATCGTGCGTGACATT 3' (SEQ ID NO:21)

3' primer 5' GATGGAGTTGAAGGTAGTTTCGTG 3' (SEQ ID NO:22)--.

IN THE CLAIMS

9. (Amended) A nucleic acid for use according to any one of the preceding claims encoding an amino acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D (SEQ ID NOs:4, 6, 8, or 10).

10. (Amended) A nucleic acid for use according to claim 9 [any one of the preceding claims] having a nucleic acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D (SEQ ID NOs:3, 5, 7 or 9).

CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

This invention relates to chimeric cell surface proteins, nucleic acids encoding such proteins, and the use of these molecules in therapy such as cancer therapy which involves the selective induction of apoptosis in particular target cell types *in vivo* or *in vitro*.

Fas (APO-1, CD95) is a member of a large family of conserved transmembrane proteins known collectively as the tumor necrosis factor receptor (TNFR) family (Baker and Reddy, 1998). Upon interaction with their respective cell surface and/or soluble ligands, for example, FasL, TNF-a, LT-a, TRAIL, RANKL/TRANCE, TWEAK/Apo-3L, a subset of these proteins including, for example, Fas, TNFR1, TRAIL-R1/DR4, TRAIL-R2/DR5, OPG, TRAMP/DR3 and DR6, induce apoptosis, a form of programmed cell death characterised by a series of biochemical events that result ultimately in the degradation of genomic DNA (Baker and Reddy, 1998). Receptor oligomerization induced by ligand binding is critical to this process (Ware et al., 1996). The cytoplasmic domain of these various pro-apoptotic proteins contains a conserved amino acid sequence known as the "death domain" that upon receptor ligation associates with a homologous domain present within a number of adapter proteins, for example, FADD/MORT1, TRADD and RIP (Schulze-Osthoff *et al.*, 1998), triggering the activation of downstream caspases, leading ultimately to the induction of apoptosis (Nunez *et al.*, 1998).

Takebayashi *et al.*, (1996) describe a method in which chimeric proteins that incorporate the transmembrane and cytoplasmic

domains of murine Fas fused in-frame to a cytoplasmic ligand-binding domain derived from the rat estrogen receptor or human retinoic acid receptor, induce the apoptotic cell death of transfected L929 and HeLa cells *in vitro* following addition of the corresponding ligand (17β-estradiol or retinoic acid). Human pancreatic carcinoma cell lines transfected with a DNA construct encoding the Fas-estrogen receptor chimera were similarly killed *in vitro* in the presence of 17β-estradiol (Kawaguchi *et al.*, 1997).

Kodaira *et al* describe the replacement of the cytoplasmic ligand-binding domain in the chimeric protein described above with an equivalent domain derived from a mutant estrogen receptor, generating a fusion that is unable to bind estrogen, but which retains affinity for the synthetic estrogen agonist 4-hydroxytamoxifen. L929 cells transfected with a DNA construct encoding this chimeric protein were killed *in vitro* in the presence of tamoxifen but not in the presence of 17β-estradiol (Kodaira *et al.*, 1998).

Although the constructs described above may have utility in cancer gene therapy, they lack specificity for tumor cells. Normal tissues that express the chimeric protein will also be killed in the presence of the appropriate ligand, which in an *in vivo* setting would preferably be administered systemically. Moreover, the design of these chimeric proteins and, in particular, the cytoplasmic location of the ligand-binding domain, limits the range of potential ligands to those capable of crossing the cell membrane, for example, lipophilic hormones.

In studies designed to investigate the nature of the signal transduction events triggered via hemopoietic growth factor receptors, Takahashi *et al.*, (1996) describe chimeric proteins in which the extracellular ligand-binding domain of the murine G-CSF receptor was fused to the cytoplasmic domain of murine Fas. Importantly, however, when expressed in the mouse T cell line WR19L or the myeloid cell line FDC-P1, such chimeric receptors did not induce cell death when dimerized by interaction with G-CSF. Cell death could be induced by treatment of transduced cells with a polyvalent anti-G-CSF receptor antibody suggesting that oligomerization is necessary to activate the apoptotic process. Such studies indicate that homodimeric cytokines, such as VEGF, do not induce cell death upon interaction with the corresponding receptor-Fas chimera.

Crabtree et al (US 5,834,266 and US 5,994,313) describe a procedure for the regulated (inducible) dimerization or oligomerization of intracellular proteins and disclose the use of this procedure to regulatably initiate cell-specific apoptosis (programmed cell death) in genetically engineered cells. Chimeric proteins are disclosed which contain a portion of the cytoplasmic domain of Fas or the TNF receptor and induce apoptotic cell death upon oligomerization with appropriate ligands. Polypeptide ligands proposed for inducing the cross-linking of the chimeric protein are either membrane permeable or have molecular weights of less than 5 kD. Cellular specificity may be achieved in the Crabtree et al procedure through the use of promoter elements or other regulatory sequences that restrict expression of the chimeric protein to particular cell types in vitro or in vivo.

The investigations described herein relate to the expression and/or functional activity of various cell surface receptors which are altered during the malignant process. The expression of both cell surface and soluble ligands may also be induced within the tumor micro-environment. While such changes may contribute to tumor growth, local invasion and metastasis, they also offer opportunities for therapeutic intervention.

The present invention relates to the unexpected discovery that these cellular changes may allow the specific targeting of particular cells in methods of gene therapy.

One aspect of the present invention therefore provides an isolated nucleic acid encoding a polypeptide comprising;

(i)an extra-cellular domain which binds multivalent ligand preferentially at the surface of a target cell relative to a non target cell,

(ii) a membrane spanning domain, and

(iii) a cytoplasmic domain which induces cell death in a target cell upon binding of the extra-cellular domain with the multivalent ligand.

Binding of the extra-cellular domain and the multivalent ligand may be directed preferentially to the surface of target cells by employing as an extracellular domain in the chimeric polypeptide, a ligand-binding domain from a receptor which is preferentially activated in a target cell i.e. the receptor is

more active in binding ligand on a target cell than on a non target cell.

Preferential binding of the extra-cellular domain and the ligand may alternatively or additionally be achieved at the surface of a target cell relative to a non-target cell by employing an extracellular domain from a receptor whose ligand is preferentially expressed in the vicinity of a target cell i.e. is found in high concentration at or near the target cell relative to elsewhere.

Polypeptide encoded by nucleic acid of the present invention herein represents a further aspect of the present invention.

A polypeptide of the present invention may therefore include;

(i)an extra-cellular domain which binds multivalent ligand preferentially at the surface of a target cell relative to a non target cell,

(ii) a membrane spanning domain, and

(iii) a cytoplasmic domain which induces cell death in a target cell upon binding of the extra-cellular domain with the multivalent ligand.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except

possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. The coding sequence shown herein is a DNA sequence. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as encompassing reference to the RNA equivalent, with U substituted for T.

Nucleic acid of the present invention may be operably linked to a regulatory element on an expression vector. Suitable expression vectors include plasmids, retroviral vector, adenoviral vector, adeno-associated viral vector.

Nucleic acid may be provided as part of a replicable vector, and also provided by the present invention are a vector including nucleic acid as set out above, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*. Regulatory sequences may allow also expression in human cell types, particularly human cell types whose selective destruction would have therapeutic benefits.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter

sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Nucleic acid according to the present invention may be used in methods of gene therapy, for instance in treatment of individuals with the aim of curing (wholly or partially) cancer, autoimmune disease, inflammation, psoriasis and other conditions requiring selective destruction of particular cell types. This may ease one or more symptoms of the disease.

Soluble ligands which may interact with extra-cellular domains of polypeptides of the present invention may include proteins such as growth factors, for example, VEGF, EGF and PDGF. Suitable ligands may also include one or more glycosaminoglycans such as, for example hyaluronan and chondroitin-4-sulfate. Generally, ligands suitable for use according to the present invention may be endogenous proteins of molecular weight greater than 5kD or glycosaminoglycans and are not membrane permeable

The ligand which interacts with the extra cellular domain may be produced by the target cells themselves or by

cells in the vicinity of the target cells such that the target cells are in contact with a concentration of the ligand sufficient to induce dimerisation or oligomerisation of the extracellular domain. Crosslinking of the encoded chimeric cell surface protein by an appropriate multivalent ligand binding to the extracellular domain induces the apoptotic death of cells expressing the chimeric protein product.

Target cells may be of any cell type which is desirably destroyed selectively in a method of therapeutic treatment, for example treatment of cancer, auto-immune disease, inflammation and psoriasis. Suitable target cells may be selected from tumour cells, endothelial cells, smooth muscle cells, fibroblasts and hemopoietic cells.

A variety of extracellular ligand-binding domains, and cytoplasmic "death domains" may be employed in the practice of the present invention. (See Hofmann K. and Tschopp, J. 1995, for review of "death domains").

The extracellular domain of the chimeric protein should be differentially active on target cells relative to non-target cells (i.e. more active on target cells) or should be capable of binding an endogenous multivalent ligand which is differentially expressed in the vicinity of target cells relative to non-target cells (i.e. higher expression near target cells).

A suitable extracellular domain for use in chimeric polypeptides according to the present invention may include an extracellular domain of CD44 (cluster of differentiation 44, Stamenkovic et al 1989, Accession No: M24915), ICAM-1 (intercellular adhesion molecule - 1, Staunton et al 1988, Accession No:J03132), VEGFR1/Flt-1 (vascular endothelial growth factor receptor 1,Shibuya et al 1990, Accession No: NM_002019), VEGFR2/KDR/Flk-1 (vascular endothelial growth factor receptor 2,Patterson et al 1995, Accession No: AF035121), VEGFR3/Flt-4 (fms related tyrosine kinase 4, Galland et al 1992, Galland et al 1993, Accession No: NM_002020), PDGFRα (platelet derived growth factor receptor alpha, Matsui et al 1989, Accession No: NM_006206), PDGFRβ (platelet derived growth factor receptor beta, Gronwald et al 1988, Accession No: NM_002609) and EGF receptor (epidermal growth factor receptor (avian erythroblastic leukaemia viral homologue (v-erb-b) oncogene homolog) Ullrich et al 1984 Acession number NM_005228) or other related receptors.

The extracellular domain of the chimeric polypeptide may comprise the complete extracellular domain of a receptor protein or a portion or fragment thereof which retains the ability to induce oligomerisation of the chimeric polypeptide on binding to ligand.

The signal peptide of CD44 (M24915) starts at amino acid -19 (Met) which corresponds to bases 116-118 of the published nucleotide sequence, and ends at amino acid -1 (Leu), which corresponds to bases 170-172 of the nucleotide sequence. The extracellular domain of CD44

starts at amino acid +1 (Ala) which corresponds to bases 173-175 of the nucleotide sequence, and ends at amino acid +249 (Glu), which corresponds to bases 917-919 of the nucleotide sequence.

The signal peptide of ICAM-1 (J03132) starts at amino acid -27 (Met) which corresponds to bases 58-60 of the published nucleotide sequence, and ends at amino acid -1 (Ala), which corresponds to bases 136-138 of the nucleotide sequence. The extracellular domain of ICAM-1 starts at amino acid +1 (Gln) which corresponds to bases 139-141 of the nucleotide sequence, and ends at amino acid +453 (Glu), which corresponds to bases 1495-1497 of the nucleotide sequence.

The signal peptide of FLT-1 (MN_002019) starts at amino acid -22 (Met) which corresponds to bases 250-252 of the nucleotide sequence, and ends at amino acid -1 (Gly), which corresponds to bases 313-315 of the nucleotide sequence. The extracellular domain of FLT-1 starts at amino acid +1 (Ser) which corresponds to bases 316-318 of the nucleotide sequence, and ends at amino acid +736 (Glu), which corresponds to bases 2521-2523 of the nucleotide sequence.

The signal peptide of FLK-1(AF035121) starts at amino acid -19 (Met) which corresponds to bases 304-306 of the published nucleotide sequence, and ends at amino acid -1 (Ala), which corresponds to bases 358-360 of the nucleotide sequence. The extracellular domain of FLK-1 starts at amino acid +1 (Ala) which corresponds to bases

361-363 of the nucleotide sequence, and ends at amino acid +745 (Glu), which corresponds to bases 2593-2595 of the nucleotide sequence.

The signal peptide of EGF (NM_005228) starts at amino acid -24 (Met) which corresponds to bases 187-189 of the published nucleotide sequence, and ends at amino acid -1 (Ala), which corresponds to bases 256-258 of the nucleotide sequence. The extracellular domain of EGF starts at amino acid +1 (Leu) which corresponds to bases 259-261 of the nucleotide sequence, and ends at amino acid +612 (Ser), which corresponds to bases 2119-2121 of the nucleotide sequence.

The signal peptide of PDGF Receptor beta (NM_002609) starts at amino acid -32 (Met) which corresponds to bases 357-359 of the published nucleotide sequence, and ends at amino acid -1 (Gly), which corresponds to bases 450-452 of the published nucleotide sequence. The extracellular domain of PDGF Receptor beta starts at amino acid +1 (Leu) which corresponds to bases 453-455 of the nucleotide sequence, and ends at amino acid +499 (Lys), which corresponds to bases 1947-1949 of the nucleotide sequence.

The signal peptide of PDGF Receptor alpha (NM_006206) starts at amino acid -24 (Met) which corresponds to bases 140-142 of the published nucleotide sequence, and ends at amino acid -1 (Gln), which corresponds to bases 209-211 of the published nucleotide sequence. The extracellular domain of PDGF Receptor alpha starts at amino acid +1

(Leu) which corresponds to bases 212-214 of the nucleotide sequence, and ends at amino acid +500 (Glu), which corresponds to bases 1709-1711 of the nucleotide sequence.

The signal peptide of FLT-4 (NM_002020) starts at amino acid -22 (Met) which corresponds to bases 22-24 of the published nucleotide sequence, and ends at amino acid -1 (Val), which corresponds to bases 85-87 of the published nucleotide sequence. The extracellular domain of FLT-4 starts at amino acid +1 (Ser) which corresponds to bases 88-90 of the nucleotide sequence, and ends at amino acid +753 (Glu), which corresponds to bases 2344-2346 of the published nucleotide sequence.

The cytoplasmic domain of the expressed polypeptide may comprise a "death domain" from a member of the Fas/TNFR family, preferably the cytoplasmic domain from a receptor protein which is member of the Fas/TNFR family, more preferably the cytoplasmic domain from Fas.

The death domain of the human Fas/Apo-1/CD95 (Protein Database Accession No: P25445) consists of amino acid residues 230 to 314 and has the following sequence;

KYITTIAGVM TLSQVKGFVR KNGVNEAKID EIKNDNVQDT AEQKVQLLRN WHQLHGKKEA YDTLIKDLKK ANLCTLAEKI QTII

The death domain of the human TNFR1 (Protein Database Accession No: P19438) consists of amino acid residues 356 to 441 and has the following sequence;

PATLY AVVENVPPLR WKEFVRRLGL SDHEIDRLEL QNGRCLREAQ YSMLATWRRR TPRREATLEL LGRVLRDMDL LGCLEDIEEA L.

Introduction of nucleic acid into a cell may take place *in vivo* by way of gene therapy, as discussed below. A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken.

Supplementary targeting therapies may be used to deliver nucleic acid more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Supplementary targeting may be desirable to reduce still further the chimeric protein induced apoptosis of non-target cells.

A vector containing a nucleic acid of the present invention may undergo supplementary targeting to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells. For example, expression of the nucleic acid of the present invention may be placed under the control of an appropriate promoter and/or enhancer element that is functional in the target

cell type or tissue but not in other non target cell types or tissues, or under the control of a promoter and/or enhancer element that can be induced or activated locally by an appropriate stimulus (e.g. ionising radiation).

Viral vectors may be targeted to a selected tissue or cell type using specific binding molecules, such as a sugar, glycolipid or protein such as an antibody or binding fragment thereof. Nucleic acid may be incorporated into a virion expressing a chemically or genetically altered cellular receptor that recognises a differentially expressed counter receptor on a target cell.

Nucleic acid may be targeted by means of linkage to a protein ligand (such as an antibody or binding fragment thereof) via polylysine, with the ligand being specific for a receptor present on the surface of the target cells. Vectors such as viral vectors have been used to introduce genes into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see e.g. US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including adenovirus, papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses, including gibbon ape leukaemia virus, Rous Sarcoma Virus, Venezualian equine enchephalitis virus, Moloney murine leukaemia virus and murine mammary tumourvirus. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

Disabled virus vectors are produced in helper cell lines in which genes required for production of infectious viral particles are expressed. Helper cell lines are generally missing a sequence which is recognised by the mechanism which packages the viral genome and produce virions which contain no nucleic acid. A viral vector which contains an intact packaging signal along with the gene or other sequence to be delivered (e.g. encoding the chimeric polypeptide) is packaged in the helper cells into infectious virion particles, which may then be used for the gene delivery.

Other known methods of introducing nucleic acid into cells include electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer. Liposomes can encapsulate RNA, DNA and virions for delivery to cells. Depending on factors such as pH, ionic strength

and divalent cations being present, the composition of liposomes may be tailored for targeting of particular cells or tissues. Liposomes include phospholipids and may include lipids and steroids and the composition of each such component may be altered. Targeting of liposomes may also be achieved using a specific binding pair member such as an antibody or binding fragment thereof, a sugar or a glycolipid.

The aim of gene therapy using nucleic acid encoding the chimeric polypeptide, is to generate the expression product of the nucleic acid in cells. In target cell types, endogenously produced ligand binds the extra-cellular domain of chimeric protein setting off a series of biochemical events leading to the apoptotic death of the target cell. Such treatment may be therapeutic or prophylactic, for example in the treatment of cancer, auto-immune disease, inflammation or psoriasis.

Administration of a nucleic acid molecule according to the present invention to an individual, is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

Thus, the present invention extends in various aspects to a pharmaceutical composition, medicament, drug or other composition comprising a nucleic acid as described above, a method comprising administration of such a composition to a patient, e.g. for expressing chimeric polypeptide for instance in treatment of treatment of cancer, auto-immune disease, inflammation or psoriasis or other disease, use of such a substance in manufacture of a composition for administration, e.g. for expressing chimeric polypeptide for instance in treatment of treatment of cancer, auto-immune disease, inflammation or psoriasis or other disease, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an

adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

An aspect of the present invention therefore provides a nucleic acid or expression vector as described herein for use in methods of treatment of cancer, autoimmune disease, inflammation, psoriasis and other conditions requiring selective destruction of particular cell types.

A nucleic acid or expression vector as described herein may also be used in the manufacture of a medicament for the treatment of cancer, autoimmune disease, inflammation, psoriasis and other normal or abnormal conditions requiring selective destruction of particular cell types.

This aspect of the present invention also provides the use of a nucleic acid or expression vector as described herein in a method of treatment of cancer, autoimmune disease, inflammation, psoriasis and other conditions requiring selective destruction of particular cell types.

Nucleic acids as described herein may be administered as a sole therapy or in combination with other therapies, either simultaneously or sequentially dependent upon the condition to be treated. For the treatment of solid tumours, nucleic acid of the present invention may be administered in combination with radiotherapy or photodynamic therapy, or in combination with other nucleic acid constructs or anti-tumour substances including mitotic inhibitors, for example, vinblastine, paclitaxel and docetaxel; alkylating agents, for example, cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example, 5-flurourocil, cytosine arabinoside and hydroxyurea; intercalating agents, for example, adriamycin and bleomycin; enzymes, for example, aspariginase; topoisomerase inhibitors, for example, etoposide, topotecan and irinotecan; thymidine synthase inhibitors, for example, raltitrexed; vascular-targeting agents, for example, combretastatin A4 disodium phosphate; biological response modifiers, for example, interferon; antibodies, for example, edrecolomab; and hormone agonists, for example, tamoxifen. Such combination treatment may involve simultaneous or sequential application of the individual components of the treatment.

A convenient way of producing a polypeptide of the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

A further aspect of the present invention therefore provides a method of producing a polypeptide comprising;

introducing a nucleic acid as described herein into a host cell,

causing or allowing expression of said nucleic acid to produce a polypeptide.

A further aspect of the present invention provides a method for inducing the apoptotic cell death of target cells comprising;

introducing a nucleic acid as described herein into a target cell,

causing or allowing expression of said nucleic acid to produce a polypeptide; and,

contacting said polypeptide with a ligand which interacts with said polypeptide,

said interaction causing apoptotic death of said target cell.

Methods according to the present invention may be performed in vitro, for example using a mammalian cell line as a target cell. Suitable cell lines include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and COS cells. Many other suitable cell lines are known in the art. Ligand may be endogenously produced by the cell line or may be exogenous ligand added to the culture medium.

One embodiment of the present invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another pro-apoptotic member of the Fas/TNFR family, and the extracellular ligand-binding domain of the adhesion protein CD44.

CD44 is a broadly distributed cell surface glycoprotein that can function as a receptor for a variety of extracellular matrix and cell surface ligands including, for example, the glycosaminoglycans hyaluronan and chondroitin-4-sulfate (Lesley *et al.*, 1993; Cooper and Dougherty, 1995; Chiu *et al.*, 1999). In common with many other adhesion proteins, however, the ligand binding function of CD44 is not regulated simply by expression (Lesley *et al.*, 1993). Thus, while many normal cell types

express CD44, only a subset of these can bind either immobilised or soluble hyaluronan (Lesley *et al.*, 1993). The hyaluronan binding function of CD44 is activated by various stimuli and is frequently induced on malignant cells (Lesley and Hyman, 1992; Lesley *et al.*, 1993; Lesley *et al.*, 1997; Sy *et al.*, 1997). While the precise mechanism involved has not yet been defined, evidence suggests that changes in the glycosylation of CD44 may be important in regulating the functional activity of the molecule (English *et al.*, 1998). For certain tumors, a correlation has been noted between CD44 expression, hyaluronan-binding function, or the expression of particular alternatively spliced CD44 isoforms, and metastatic propensity and/or poor prognosis (Cooper and Dougherty, 1995; Lesley *et al.*, 1997; Rudzki and Jothy, 1997; Sy *et al.*, 1997; Goldbrunner *et al.*, 1998; Takahashi *et al.*, 1999).

Thus, nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain derived from CD44 may be therapeutically useful, for example, in the treatment of cancer or other conditions where the destruction of cells in which CD44 is activated, is desired. It is noteworthy that the CD44 ligand hyaluronan is also differentially expressed in various tissues and that production of the molecule may be upregulated at sites of angiogenesis, inflammation, wound healing, and within certain solid tumors (Laurent and Fraser, 1992; Oksala *et*

al., 1995; Rooney *et al.*, 1995; Fraser *et al.*, 1997; Setala et al., 1999).

A second embodiment of the present invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and an extracellular ligand-binding domain derived from the adhesion protein ICAM-1. The cell surface glycoprotein ICAM-1 functions as a ligand for the β2-integrin LFA-1 (van de Stolpe and van der Saag, 1996). Expression of ICAM-1 is induced on endothelial cells at sites of inflammation and within tumours as a result of exposure to various pro-inflammatory cytokines (Walsh and Murphy, 1992; van de Stolpe and van der Saag, 1996). Nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and the extracellular domain derived from ICAM-1 may be useful in circumstances where the selective destruction of target cells in the presence of hemopoietic cells expressing LFA-1 is desirable. For example, this method could be used to effect the killing of endothelial cells within tumours or at sites of inflammation. It is noteworthy that although LFA-1 is widely expressed on hemopoietic cells, the ligand-binding function of the molecule is only induced following appropriate stimulation (Springer, 1990).

A third embodiment of the present invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived

from Fas or another member of the Fas/TNFR family, and an extracellular ligand-binding domain derived from a receptor for the cytokine vascular endothelial growth factor (VEGF), for example VEGFR1/Flt-1, VEGFR2/KDR/Flk-1 or VEGFR3/Flt-4 (Neufeld *et al.*, 1999). Various soluble ligands are known to be induced within both normal and malignant tissues in response to specific microenvironmental stimuli. Thus, the chaotic nature of the angiogenic process that occurs within solid tumours generates regions of chronic and transient hypoxia not found in normal tissues (Chaplin and Trotter, 1990; Vaupel, 1996; Brown and Giaccia, 1998).

Exposure to hypoxic conditions can induce tumour cells to produce soluble mediators such as VEGF that function to induce the formation of new blood vessels (Shweiki *et al.*, 1992; Minchenko *et al.*, 1994). Nucleic acid encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and an extracellular ligand-binding domain derived from a VEGF receptor, for example, VEGFR1/Flt-1, VEGFR2/KDR/Flk-1 or VEGFR3/Flt-4, may be therapeutically useful in circumstances where the destruction of normal or malignant target cells in the presence of VEGF is desirable.

A fourth embodiment of the present invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and an extracellular ligand-binding domain derived from a

receptor for the cytokine platelet-derived growth factor (PDGF). Restenosis is a significant clinical problem associated with the trauma induced by mechanical procedures such as coronary angioplasty and stenting that are commonly used in the treatment of vascular occlusive disease.

Vascular smooth muscle cell proliferation plays a critical role in the development of these conditions and in the evolution of spontaneous atherosclerosis, hypertension-related arteriosclerosis, and venous bypass graft arteriosclerosis (Zou *et al.*, 1998). PDGF, is a potent chemotactic and mitogenic agent for vascular smooth muscle cells and recent studies have implicated this molecule in the development of these various vascular lesions (Abe *et al.*, 1998). Thus, nucleic acid encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and an extracellular domain derived from the alpha or beta PDGF receptors, may be therapeutically useful, for example, in the treatment of atherosclerosis or restenosis, or other conditions where the destruction of target cells in the presence of PDGF is desirable.

A fifth embodiment of the present invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and an extracellular ligand-binding domain derived from a receptor for the cytokine epidermal growth factor (EGF).

Members of EGF superfamily including, for example, EGF and Cripto-1, play an important role in regulating the proliferation and differentiation of both normal and malignant epithelial cells (Jones *et al.*, 1999; Salomon *et al.*, 1999; Thomas *et al.*, 1999). High levels of EGF and related proteins can be detected within various solid malignancies including, for example, those of the breast, ovary and stomach. EGF induces homodimerization of the EGF receptor (EGFR) and heterodimerization of the EGFR and ErbB2 (Wang *et al.*, 1999).

Thus, nucleic acid encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and the extracellular ligand-binding domain of an EGF receptor, may be therapeutically useful, for example, in the treatment of epithelial malignancies, or other conditions where the destruction of target cells in the presence of EGF is desirable.

Nucleic acid sequences encoding the ligand-binding domains and counter-receptors discussed above represent exemplary domains useful in the practice of the present invention. It will be appreciated, however, that following the teachings and guidance of the present specification, one of skill in the art may select other sequences suitable for use with the present invention, and that the use of such sequences is considered to be within the scope of the present invention.

Those skilled in the art will also recognise that cytoplasmic domains derived from the various members of the Fas/TNFR family may have different activities in different target cell types. It will also be appreciated that cytoplasmic domains lacking classical "death domains" of the type seen in the various members of the Fas/TNFR family and which kill cells by a different mechanism may nevertheless prove suitable for use with the present invention

The following examples illustrate but are in no way are intended to limit the present invention, and that the use of such sequences is considered to be within the scope of the present invention.

The examples will be described with reference to the following figures;

Figure 1 shows the pCEP4 expression vector with the alternative cDNA inserts used herein.

Figures 2A and 2B show the nucleic acid and predicted amino acid sequence of $C44H^{EXTRA}$-$FAS^{TM/CYTO}$

Figures 3A and 3B show the nucleic acid and predicted amino acid sequence of $C44H^{EXTRA/TM}FAS^{CYTO}$

Figure 4 shows the results of FACS analysis of the expression of Fas, CD44H and chimeric proteins $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ and $C44H^{EXTRA/TM}FAS^{CYTO}$ on the surface of transfected K562 cells.

Figure 5 shows the apoptosis (expressed as % hypodiploid) of K562 cells transfected with CD44H , $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ or $C44H^{EXTRA/TM}FAS^{CYTO}$ upon adhesion to hyaluronan.

Figure 6 shows the inhibition of the clonogenic potential of ECV304 cells when transfected with $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ or $C44H^{EXTRA/TM}FAS^{CYTO}$.

Figure 7 shows the inhibition of the clonogenic potential of QBI-293 cells when transfected with $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ or $C44H^{EXTRA/TM}FAS^{CYTO}$.

Figure 8 shows the inhibition of the clonogenic potential of MCF-7 cells when transfected with $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ or $C44H^{EXTRA/TM}FAS^{CYTO}$.

Figure 9 shows the inhibition of the clonogenic potential of PC-3 cells when transfected with $C44H^{EXTRA}$-$FAS^{TM/CYTO}$ or $C44H^{EXTRA/TM}FAS^{CYTO}$.

Figures 10A to 10D show the nucleic acid and amino acid sequence of $Flt\text{-}1^{EXTRA}Fas^{TM/CYTO}$.

Figures 11A to 11D show the nucleic acid and amino acid sequence of $Flt\text{-}1^{EXTRA/TM}Fas^{CYTO}$.

Figure 12 shows the expression of $Flt\text{-}1^{EXTRA}Fas^{TM/CYTO}$ and $Flt\text{-}1^{EXTRA/TM}Fas^{CYTO}$ on the surface of K562 cells using FACS analysis.

Figure 13 shows the inhibition of the clonogenic potential of ECV304 cells when transfected with Flt-$1^{EXTRA}Fas^{TM/CYTO}$ or Flt-$1^{EXTRA/TM}Fas^{CYTO}$.

Figure 14 shows the inhibition of the clonogenic potential of QBI-293 cells when transfected with Flt-$1^{EXTRA}Fas^{TM/CYTO}$ or Flt-$1^{EXTRA/TM}Fas^{CYTO}$.

Figure 15 shows the inhibition of the clonogenic potential of MCF-7 cells when transfected with Flt-$1^{EXTRA}Fas^{TM/CYTO}$ or Flt-$1^{EXTRA/TM}Fas^{CYTO}$.

EXAMPLES

Example 1

Cytotoxic activity of CD44-Fas chimeric proteins

Vector Construction

A full length CD44H cDNA was isolated from pCDM8.CD44H clone 2.3 (Dougherty *et al*, 1991) by digestion with HindIII and NotI and the fragment obtained cloned into the HindIII-NotI sites of the episomal expression vector pCEP4 (Invitrogen) generating a plasmid designated pCEP4.CD44H. The major features of this vector are shown in Figure 1. Digestion of pCEP4.CD44H with XhoI released a fragment containing the full length CD44H cDNA, which was blunted using T4 DNA polymerase and cloned into the EcoRV site of pZErO2 (Invitrogen). Orientation of the insert was determined by digestion with a panel of

restriction enzymes and an appropriate clone digested with EcoRI and NotI to release the full length CD44H cDNA in which the 3′ end of the gene is located adjacent to the EcoRI site. This fragment was cloned into the EcoRI-NotI sites of pBluescript (KS+) (Stratagene) generating a vector designated pBS.CD44H.

mRNA was isolated from approximately 4 x 10^7 Jurkat cells using the Stratagene mRNA Isolation Kit (Stratagene). The mRNA was reverse transcribed and cDNA synthesized using the Pharmacia cDNA Synthesis Kit (Pharmacia) as per the manufacturers instructions using random hexanucleotide primers. A 'full-length' human Fas cDNA (Fas^{FL}) or cDNA fragments encoding the transmembrane and cytoplasmic domains of human Fas ($Fas^{TM/CYTO}$) or only the cytoplasmic domain of the molecule (Fas^{CYTO}) were generated by polymerase chain reaction (PCR) using the following primer pairs designed on the basis of published Fas sequences (Itoh *et al.*, 1991).

Fas^{FL}

5′ primer 5′ GCG**GAATTC**AGGGGCGGGCACTGGCAC 3′

EcoR1

3′ primer 5′ GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3′

Xho1

$Fas^{TM/CYTO}$

5′ primer 5′ AACG**TGATCA**TCCTTTGTCTTCTTCTTTTG 3′

BclI

3′ primer 5′ GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3′

XhoI

Fas^{CYTO}

5′ primer 5′ G**CCCGGG**GTGAAGAGAAAGGAAGTACAG 3

SmaI

3′ primer 5′ GG**CTCGAG**AATCTTTTCAAACACTAATTGC 3′

XhoI

The underlined base pairs in the 5′ $Fas^{TM/CYTO}$ primer are not found in Fas and were introduced to maintain the correct reading frame. The restriction enzyme sites used in subsequent cloning steps are indicated in bold.

PCR reactions (94°C for 30s, 50°C for 30s and 72°C for 1 min; 40 cycles) were carried out in an OmniGene Thermacycler (Hybaid) using Ampli-Taq (Perkin-Elmer). PCR products were blunted using T4 DNA polymerase and cloned into the EcoRV site of pZErO2 generating vectors designated pZErO2.Fas^{FL}, pZErO2.$Fas^{TM/CYTO}$ and pZErO2.Fas^{CYTO}.

pZErO2.Fas^{FL} was digested with EcoRI-XhoI to release the full length Fas cDNA which was then ligated into the EcoRI-XhoI sites of pBluescript (KS+) generating the vector pBS.Fas^{FL}. Digestion of pBS.Fas^{FL} with NotI and XhoI released a fragment containing the full length Fas cDNA

which was then ligated into the corresponding NotI-XhoI sites of the episomal expression vector pCEP4 (Invitrogen). The major features of this vector, designated pCEP4.FAS^{FL}, are illustrated in Figure 1.

To generate a nucleic acid construct encoding a chimeric protein containing the extracellular domain of CD44H and the transmembrane and cytoplasmic domains of human Fas ($CD44^{EXTRA}Fas^{TM/CYTO}$), pBS.CD44H was digested with BclI and XhoI to remove the transmembrane and cytoplasmic domain of CD44H and a BclI-XhoI fragment derived from pZErO2.Fas$^{TM/CYTO}$ containing the transmembrane and cytoplasmic domains of Fas was ligated into the corresponding sites in the plasmid generating a vector designated pBS.$CD44^{EXTRA}Fas^{TM/CYTO}$. The complete nucleic acid sequence and predicted animo acid sequence of $CD44^{EXTRA}Fas^{TM/CYTO}$ are shown in Figures 2A and 2B.

To generate a nucleic acid construct encoding a chimeric protein containing the extracellular and transmembrane domains of CD44H and the cytoplasmic domain of human Fas ($CD44^{EXTRA/TM}Fas^{CYTO}$) pBS.CD44H was digested with NotI and EcoRI to release the full length CD44H cDNA which was then partially digested with HincII to obtain a NotI-HincII fragment that contained only the extracellular and transmembrane domains of the CD44H molecule. pZErO2.Fas^{CYTO} was digested with NotI and SmaI and the NotI-HincII fragment containing the extracellular and transmembrane domains of CD44H was ligated into the plasmid generating a vector designated pZErO2.$CD44^{EXTRA/TM}Fas^{CYTO}$. The complete

nucleic acid sequence and predicted amino acid sequence of $CD44^{EXTRA/TM}Fas^{CYTO}$ are shown in Figure 3A and 3B.

In order to test the functional activity of the nucleic acid constructs, full length $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ chimeric cDNAs were isolated by digestion of pBS.$CD44^{EXTRA}Fas^{TM/CYTO}$ and pZErO2.$CD44^{EXTRA/TM}Fas^{CYTO}$ with NotI and XhoI. The fragments obtained were then cloned into the NotI-XhoI sites of the episomal expression vector pCEP4 (Invitrogen) generating vectors designated pCEP4.$CD44^{EXTRA}Fas^{TM/CYTO}$ and pCEP4.$CD44^{EXTRA/TM}Fas^{CYTO}$. The major features of both plasmids are illustrated in Figure 1.

Cell lines and culture conditions

The human cell lines K562 (erythroleukemia), ECV304 (a variant of the T28 bladder carcinoma), MCF-7 (breast adenocarcinoma) and PC-3 (prostatic adenocarcinoma),were obtained from the American Type Culture Collection (ATCC). QBI-293 (adenovirus 5 transformed kidney epithelial cells) was obtained from Quantum Biotechnology Inc. All tumor cell lines except MCF-7 were maintained at 37°C in an atmosphere containing 5% CO_2 in Dulbecco's Minimal Essential Medium (DMEM) supplemented with fetal bovine serum (10%), L-glutamine (2mM), penicillin (50 units/ml), and streptomycin sulfate (50 mg/ml). MCF-7 was maintained in Eagles Minimum Essential Medium (EMEM) supplemented with fetal bovine serum (10%), bovine insulin (0.01 mg/ml), glutamine (2mM), non-essential amino acids (0.1 mM) sodium pyruvate (1.0 mM), penicillin (50 units/ml), and streptomycin sulfate (50 mg/ml).

Cell surface expression of $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ chimeric proteins in transfected cell lines

K562 cells do not express CD44 and can be used to characterize the expression and functional activity of chimeric CD44-Fas proteins in the absence of a contribution from the endogenous CD44 protein. K562 cells were transfected with plasmid DNA by electroporation using the BTX ECM 600 Electroporator System (BTX). Briefly, log-phase K562 cultures were harvested and the cells resuspended in phosphate buffered saline (PBS) at a final concentration of 1 x 10^7 cells/ml. 15 mg of plasmid DNA (pCEP4, $pCEP4.Fas^{FL}$, pCEP4.CD44H, $pCEP4.CD44^{EXTRA}Fas^{TM/CYTO}$ or $pCEP4.CD44^{EXTRA/TM}Fas^{CYTO}$) were added to a 400 ml aliquot of each cell suspension, transferred to a 2 mm gap cuvette and electroporated at resistance setting R3-48 ohms, 280 V, and 500 mF. The time constants obtained generally ranged from 3.0-4.0 ms. Immediately after electroporation, the transfected cells were resuspended in 30 ml tissue culture medium, plated in a 15 cm Integrid dish and incubated at 37°C in an atmosphere containing 5% CO_2. Hygromycin B (Sigma) was added 24-48 hours after electroporation at a final concentration of 250 mg/ml and the transfected cells selected for a minimum of 14 days before being further analysed.

Expression of Fas, CD44H and chimeric $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ proteins on the surface of transfected K562 cells was determined by Fluorescent Antibody Cell Sorter (FACS) analysis. Briefly, $5x10^5$ cells were

incubated with anti-CD44 mAb 4A4 tissue culture supernatant (Droll *et al.*, 1995), or the mouse anti-human Fas mAb DX2 (PharMingen) at a final concentration of 5 mg/ml or media alone, for 30 min at 4°C. After 3 washes with ice-cold Hank's balanced salt solution (HBSS) containing 2% FCS (HBSS+2% FCS), the cells were stained for a further 30 min at 4°C with an FITC-conjugated goat anti-mouse antibody (PharMingen) at a final concentration of 5 mg/ml in HBSS+2% FCS. Following extensive washing, cells were resuspended in HBSS+2% FCS containing 1 mg/ml propidium iodide (PI) (Sigma) to facilitate the identification and exclusion of dead cells, and analyzed on a FACSCalibur (Becton Dickinson). As shown in Figure 4, Fas, CD44H and the chimeric proteins $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ are all expressed at moderate to high levels on the surface of the corresponding transfected K562 cells.

Induction of apoptosis by binding to immobilized hyaluronan

Hemopoietic cells such as K562 generally produce very low or undetectable levels of hyaluronan (Laurent and Fraser, 1992; Fraser *et al.*, 1997) and K562 cells stably transfected with either $CD44^{EXTRA}Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}Fas^{CYTO}$ do not appear to exhibit a high rate of spontaneous apoptosis. In order to determine whether cells expressing chimeric $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ proteins undergo apoptosis upon ligand binding, the wells of 6 well tissue culture plates (Falcon) were coated overnight at 4°C with human placental hyaluronan (Sigma) (5mg/ml in

PBS). Unbound hyaluronan was decanted and the wells washed 5 times with PBS and twice with DMEM+10% FCS. $5x10^6$ transfected K562 cells in a final volume of 3 ml HBSS were added to each well. After incubation for 10 min at 37°C, non-adherent cells were removed by gently washing with medium. K562 cells transfected with CD44H or the chimeric proteins $CD44^{EXTRA}Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}Fas^{CYTO}$ bound avidly to the hyaluronan-coated dishes. Equivalent cells transfected with the pCEP4 vector alone or with pCEP4.Fas^{FL} did not adhere reflecting the absence of CD44 or other hyaluronan binding proteins on these cells.

The induction of apoptosis upon adhesion to hyaluronan was determined using the method of Fraker *et al.*, (1995). Briefly, transfected K562 cells that had been allowed to adhere to plastic surfaces coated with hyaluronan as described above were recovered by gentle pipetting at various time points ranging from 1.5-12 hours. Approximately 2 x 10^6 cells were then aliquoted into sample tubes (Falcon 2099, Becton Dickinson), pelleted by centrifugation at 350g for 10 min, washed once in HBSS, and then resuspended in 2 ml ice cold 70% ethanol with rapid but gentle mixing. Cells were fixed by incubation at -20°C for at least 4 h, centrifuged at 400 g for 10 min, washed once in HBSS and resuspended in 1 ml DNA staining solution (PBS, pH 7.4, containing 0.1% Triton X-100, 0.1 mM EDTA pH7.4, 0.05 mg/ml RNase A, and 50 mg/ml propidium iodide). Cells were stained for at least 4 h in the dark at room temperature and the apoptotic fraction determined by FACS analysis (FACSCalibur, Becton Dickinson). Briefly, data were collected for at least

10,000 events and FL2 histograms generated. Using the CellQuest software package (Becton Dickinson) gates were set to calculate the percentage of hypodiploid cells (i.e. those cells with a sub G_0/G_1 DNA content). As shown in Figure 5, K562 cells transfected with $CD44^{EXTRA}Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}Fas^{CYTO}$ rapidly undergo apoptosis upon adhesion to hyaluronan. In contrast, although K562 cells expressing CD44H adhered to hyaluronan, they remained largely viable even if recovered 12 hours after initial attachment.

Effect of the expression of $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ chimeric proteins on the clonogeneic potential of transfected tumor cell lines

Many adherent tumor cells constitutively produce hyaluronan, which is found associated with the cell surface bound to CD44 and perhaps other molecules forming a pericellular coat (Laurent and Fraser, 1992; Knudson *et al.*, 1996; Fraser *et al.*, 1997). In order to determine whether the introduction of chimeric $CD44^{EXTRA}Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}Fas^{CYTO}$ into such cells induces cell death in the absence of added hyaluronan via an autocrine or paracrine mechanism, ECV304, QBI-293, MCF-7 and PC-3 cells were transfected with plasmid DNA by electroporation using the BTX ECM 600 Electroporator System (BTX). Briefly, sub-confluent cultures were harvested by trypsinization and cells resuspended in phosphate buffered saline (PBS) at a final concentration of 1×10^7 cells/ml. 15 mg of plasmid DNA (pCEP4, $pCEP4.Fas^{FL}$, pCEP4.CD44H, $pCEP4.CD44^{EXTRA}Fas^{TM/CYTO}$ or $pCEP4.CD44^{EXTRA/TM}Fas^{CYTO}$) were added to a 400 ml aliquot

of each cell suspension, transferred to a 2 mm gap cuvette and electroporated using the following conditions.

ECV304:- Resistance setting R3-48 ohms, 280 V, 400-500 mF. The time constants obtained ranged from 2.7-4.0 ms.

QBI-293:- Resistance setting R4-72 ohms, 270 V, 400-450 mF. The time constants obtained ranged from 2.8-3.7 ms.

MCF-7:- Resistance setting R4-72 ohms, 270 V, 400 mF. The time constants obtained ranged from 2.9-3.2 ms.

PC-3:- Resistance setting R3-48 ohms, 280 V, 300 mF. The time constants obtained ranged from 2.0-3.1 ms.

Immediately after electroporation, transfected cells were resuspended in 30 ml tissue culture medium, plated in a 15 cm Integrid dish (Falcon) and incubated at 37°C in an atmosphere containing 5% CO_2.

Western blot analysis was used confirm expression of the various transgenes in transfected cells. Briefly, although QBI-293 cells constitutively express CD44, elevated levels of species reactive with the anti-CD44 mAb 4A4 are seen in cells transfected with pCEP4.CD44H, pCEP4.CD44^{EXTRA}Fas$^{TM/CYTO}$ or pCEP4.CD44$^{EXTRA/TM}$Fas^{CYTO}. It is

noteworthy that the chimeric CD44 proteins do not differ greatly in molecular weight from endogenous CD44. Fas^{FL} and the chimeric CD44-Fas proteins can be readily detected in transfected cells by probing blots with mAb 3D5 (Alexis Biochemicals) directed against the Fas Death Domain.

In order to determine the effect of expressing the $CD44^{EXTRA}Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}Fas^{CYTO}$ chimeric proteins on the clonogenic potential of tumor cells, Hygromycin B (Sigma) was added to cultures of transfected cells 24-48 h after electroporation at a final concentration of 250 mg/ml (ECV304) or 200 mg/ml (QBI-293, MCF-7 and PC-3). Plates were incubated undisturbed for 18-21 days after which time the tissue culture supernatant was removed and the number of colonies derived from single cells that survived the treatment, were determined after staining in a solution containing 1% (w/v) methylene blue in methanol. As shown in Figures 6-9, both $CD44^{EXTRA}Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}Fas^{CYTO}$ dramatically inhibited clonogenic potential when expressed in each of the four tumor cell lines tested. In contrast, overexpression of CD44H produced a modest and variable decrease in the number of hygromycin resistant colonies relative to cells transfected with the pCEP4 vector alone. Although transfection of ECV304 cells with $pCEP4.Fas^{FL}$, had little effect on clonogeneic potential, expression of Fas^{FL} did inhibit the growth of transfected QBI-293, MCF-7 and PC-3 cells. These findings are in agreement with previous studies that demonstrated constitutive production of FasL

by PC-3 and MCF-7 (Liu et al., 1998; Gutierrez et al., 1999).

EXAMPLE 2

Cytotoxic activity of Flt-1-Fas and Flk-1-Fas chimeric proteins

Vector Construction

mRNA was isolated from approximately 5 x 10^6 human umbilical cord vascular endothelial (HUVEC) cells using the Pharmacia QuikPrep mRNA Purification Kit (Pharmacia). mRNA was reverse transcribed and cDNA synthesized using the Pharmacia cDNA Synthesis Kit (Pharmacia) as per the manufacturers instructions using random hexanucleotide primers.

cDNAs encoding the extracellular ligand-binding domain of Flt-1 and Flk-1 were generated by polymerase chain reaction (PCR) using the following primer pairs designed on the basis of published Flt-1 and Flk-1 sequences (Shibuya et al., 1990; Terman et al., 1991; Patterson et al., 1995) and sequence information submitted to GenBank (Accession numbers NM_002019 and AF035121).

Flt-1

5′ primer 5′ GC**GGGTAC**CGCGGCCAGCGGGCCTGGCGCC 3′

Kpn1

3′ primer 5′ GGC**GGATCC**GTCCGAGGTTCCTTGAACAGTGAGG 3′

BamH1

Flk-1

5′ primer 5′ GCG**GGTACC**GCCGCCGGTCGGCGCCCGGGC 3′

Kpn1

3′ primer 5′ GGC**GGATCC**CTTTTCCTGGGCACCTTCTATTATG 3′

BamH1

PCR reactions (Flt-1: 95°C for 30s, 58°C for 30s and 72°C for 2.5 min, 35 cycles; Flk-1: 95°C for 30s, 60°C for 30s and 72°C for 2.5 min, 40 cycles) were carried out in an OmniGene Thermacycler (Hybaid) using Taq DNA polymerase (Gibco-BRL). PCR products were gel purified, blunted using T4 DNA polymerase, digested sequentially with BamHI and KpnI and the fragments obtained ligated into the BamHI-KpnI sites of pBluescript (KS+) (Stratagene) generating the vectors pBS.Flt-1^{EXTRA} and pBS.Flk-1^{EXTRA}.

In order to acquire flanking restriction sites for use in subsequent cloning steps, pBS.Flt-1^{EXTRA} and pBS.Flk-1^{EXTRA} were digested with KpnI and BamHI and DNA fragments encoding the extracellular ligand-binding domain of Flt-1 and Flk-1 were isolated and ligated into the KpnI-BamHI sites of the plasmid pZErO2 (Invitrogen) generating vectors designated pZErO2.Flt-1^{EXTRA} and pZErO2.Flk-1^{EXTRA}.

To generate a nucleic acid construct encoding chimeric proteins containing the extracellular domain of Flt-1 and

Flk-1 fused in-frame to the transmembrane and cytoplasmic domains of Fas, the vector pCEP4.Fas^{FL} (see above) was digested with XhoI, blunted with T4 DNA polymerase and then digested with BamHI releasing a fragment of approximately 600 bp containing the transmembrane and cytoplasmic domains of Fas (Fas$^{TM/CYTO}$). This fragment was cloned into the BamHI-EcoRV sites of pZErO2.Flt-1^{EXTRA} and pZErO2.Flk-1^{EXTRA} generating vectors designated pZErO2.Flt-1^{EXTRA} FAS$^{TM/CYTO}$ and pZErO2.Flk-1^{EXTRA}Fas$^{TM/CYTO}$.

In order to test the functional activity of the chimeric nucleic acid constructs, full length Flt-1^{EXTRA}Fas$^{TM/CYTO}$ and Flk-1^{EXTRA}Fas$^{TM/CYTO}$ chimeric cDNAs were isolated by digestion of pZErO2.Flt-1^{EXTRA}FAS$^{TM/CYTO}$ and pZErO2.Flk-1^{EXTRA}Fas$^{TM/CYTO}$ with KpnI and NotI and the fragments obtained ligated into the KpnI-NotI sites of pCEP4 generating the vectors pCEP4.Flt-1^{EXTRA}FAS$^{TM/CYTO}$ and pCEP4.Flk-1^{EXTRA}Fas$^{TM/CYTO}$. The complete predicted nucleic acid and amino acid sequences of Flt-1^{EXTRA}FAS$^{TM/CYTO}$ and Flk-1^{EXTRA}-Fas$^{TM/CYTO}$ are shown in Figures 10A-D and Figures 11A-D respectively.

<u>Cell surface expression of Flt-1^{EXTRA}Fas$^{TM/CYTO}$ and Flk-1^{EXTRA}Fas$^{TM/CYTO}$ chimeric proteins in transfected cell lines</u>

The pCEP4.Flt-1^{EXTRA}Fas$^{TM/CYTO}$ and pCEP4.Flk-1^{EXTRA}Fas$^{TM/CYTO}$ plasmid vectors were introduced into K562 cells by electroporation and cell surface expression of the corresponding chimeric proteins determined by FACS analysis as described in Example 1 using mAb 49560.11

directed against Flt-1 (R&D Systems) and mAb FLK-12M directed against Flk-1 (Alpha Diagnostic). As shown in Figure 12, low levels of both chimeric proteins can be detected on the surface of transfected K562 cells.

Production of VEGF by tumor cells

Expression of VEGF by tumor cell lines was determined using a semi-quantitative RT-PCR technique. Briefly, mRNA was isolated from various tumor cell lines using the Pharmacia QuikPrep mRNA Purification Kit (Pharmacia) and cDNA synthesized using the Pharmacia cDNA Synthesis Kit (Pharmacia) as per the manufacturers instructions using the NotI-dT18 primer provided. ECV304 cDNA was serially diluted and PCR reactions carried out as described below in order to identify the lowest dilution that still allowed the detection of VEGF. All cDNAs were then diluted to this level and PCR reactions (95°C for 30s, 62°C for 30s 72°C for 30s; 35 cycles) carried out using the following primer pairs designed on the basis of published sequences (Ponte et al., 1984; Keck et al., 1989) and sequence information submitted to GenBank (Accession numbers NM_001101 and M27281).

VEGF

5' primer 5' GAGACCCTGGTGGACATCTTCCAGGAGTACCC 3'

3' primer 5' GGCTCCTTCCTCCTGCCCGGCTCACCGCCTCG 3'

Actin

5′ primer 5′ GAGCGGGAAATCGTGCGTGACATT 3′

3′ primer 5′ GATGGAGTTGAAGGTAGTTTCGTG 3′

The results indicated that all four of the tumor cell lines tested (ECV304, K562, PC-3 and 293) express detectable levels of VEGF mRNA although substantially lower quantities (5-10 fold) are present within ECV304 cells. Control PCR using actin primers confirmed that equivalent amounts of cDNA were added to each reaction. Previous studies have demonstrated the production of VEGF by MCF-7 cells (Lewin et al., 1999).

Effect of the expression of Flt-$1^{EXTRA}Fas^{TM/CYTO}$ and Flk-$1^{EXTRA}Fas^{TM/CYTO}$ chimeric proteins on the clonogeneic potential of transfected tumor cell lines

The pCEP4.Flt-$1^{EXTRA}Fas^{TM/CYTO}$ and pCEP4.Flk-$1^{EXTRA}Fas^{TM/CYTO}$ plasmid vectors were introduced into tumor cells and transfectants selected in Hygromycin B exactly as described in Example 1. As shown in Figure 13, expression of Flt-$1^{EXTRA}Fas^{TM/CYTO}$ or Flk-$1^{EXTRA}Fas^{TM/CYTO}$ had little if any effect on the clonogenic potential of ECV304 cells, which produce only very low levels of VEGF, as described above. In contrast, Flk-$1^{EXTRA}Fas^{TM/CYTO}$ but not Flt-$1^{EXTRA}Fas^{TM/CYTO}$ substantially inhibited the clonogenic potential of QBI-293 cells (Figure 14). Finally, both Flt-$1^{EXTRA}Fas^{TM/CYTO}$ and Flk-$1^{EXTRA}Fas^{TM/CYTO}$ inhibited the clonogenic potential of MCF-7 cells although somewhat better killing was obtained for the Flt-1 construct (Figure 15). These studies are important as they provide evidence that the naturally

occurring form of VEGF constitutively produced by tumor cells can oligomerize the chimeric Flt-$1^{EXTRA}Fas^{TM/CYTO}$ and Flk-$1^{EXTRA}Fas^{TM/CYTO}$ molecules to an extent sufficient to trigger the induction of cell death. Differences in the activity of the chimeric proteins in different tumor cells lines reflect the relative concentration of VEGF produced by each of the lines, differences in the affinity of the two chimeric proteins for VEGF and the compounding influence of endogenous Flt-1 and Flk-1 which may interfere with the oligomerization of the chimeric molecules.

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CLAIMS:

1. An isolated nucleic acid encoding a chimeric polypeptide comprising;

(i) an extra-cellular domain which binds multivalent ligand preferentially at the surface of a target cell relative to a non-target cell,

(ii) a membrane spanning domain, and

(iii) a cytoplasmic domain which induces cell death in a target cell upon binding of the extra-cellular domain with the multivalent ligand,

for use in a method of gene therapy of an individual.

2. A nucleic acid for use according to claim 1 wherein the multivalent ligand is preferentially expressed in the vicinity of the target cell

3. A nucleic acid for use according to claim 1 wherein the binding of the extra-cellular domain is preferentially activated at the surface of a target cell relative to a non target cell.

4. A nucleic acid for use according to any one of the preceding claims wherein the target cell is selected from tumour cells, endothelial cells, smooth muscle cells, fibroblasts and hemopoietic cells.

5. A nucleic acid for use according to any one of the preceding claims wherein the cytoplasmic domain comprises a "death domain" from a member of the Fas/TNFR family.

6. A nucleic acid for use according to claim 5 wherein the cytoplasmic domain comprises the cytoplasmic domain from a receptor protein which is member of the Fas/TNFR family.

7. A nucleic acid for use according to claim 6 wherein the receptor protein is Fas.

8. A nucleic acid for use according to any one of the preceding claims wherein the extracellular domain is a VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, VEGFR3/Flt-4, CD44, ICAM-1, PDGFRα, PDGFRβ or EGF receptor extracellular domain.

9. A nucleic acid for use according to any one of the preceding claims encoding an amino acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D.

10. A nucleic acid for use according to claim 9 any one of the preceding claims having a nucleic acid sequence as shown in any one of Figures 2A, 2B, 3A, 3B, 10A to 10D and 11A to 11D.

11. An expression vector comprising a nucleic acid according to any one of claims 1 to 10 operably linked to a regulatory element, for use in a method of gene therapy of an individual.

12. An expression vector for use according to claim 11 wherein the regulatory element is functional in a target cell type and not functional in a non-target cell type.

13. A expression vector for use according to claim 11 or claim 12 wherein the regulatory element is inducible.

14 A pharmaceutical composition comprising a nucleic acid according to any one of claims 1 to 10 or an expression vector according to any one of claims 11 to 13 and a pharmaceutically acceptable excipient.

15. A nucleic acid for use according to any one of claims 1 to 10 or an expression vector for use according to any one of claims 11 to 13 wherein said method of gene therapy is for the treatment of cancer, autoimmune disease, inflammation, psoriasis or other condition requiring selective destruction of a particular cell type.

16. Use of a nucleic acid according to any one of claims 1 to 10, an expression vector according to any one of claims 11 to 13 or a pharmaceutical composition according to claim 14 in the manufacture of a medicament for use in the treatment of cancer, autoimmune disease, inflammation, psoriasis or other condition requiring selective destruction of a particular cell type.

17. A method of treatment comprising administering nucleic acid according to any one of claims 1 to 10, an expression vector according to any one of claims 11 to 13 or a pharmaceutical composition according to claim 14 to an individual.

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(54) Title: CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

(57) Abstract: Chimeric cell-surface proteins are described which may be used in the selective induction of apoptosis in particular target cell types such as cancer cells *in vivo* or *in vitro*. Nucleic acid sequences encoding such proteins and methods of use relating to cancer and other therapies are provided.

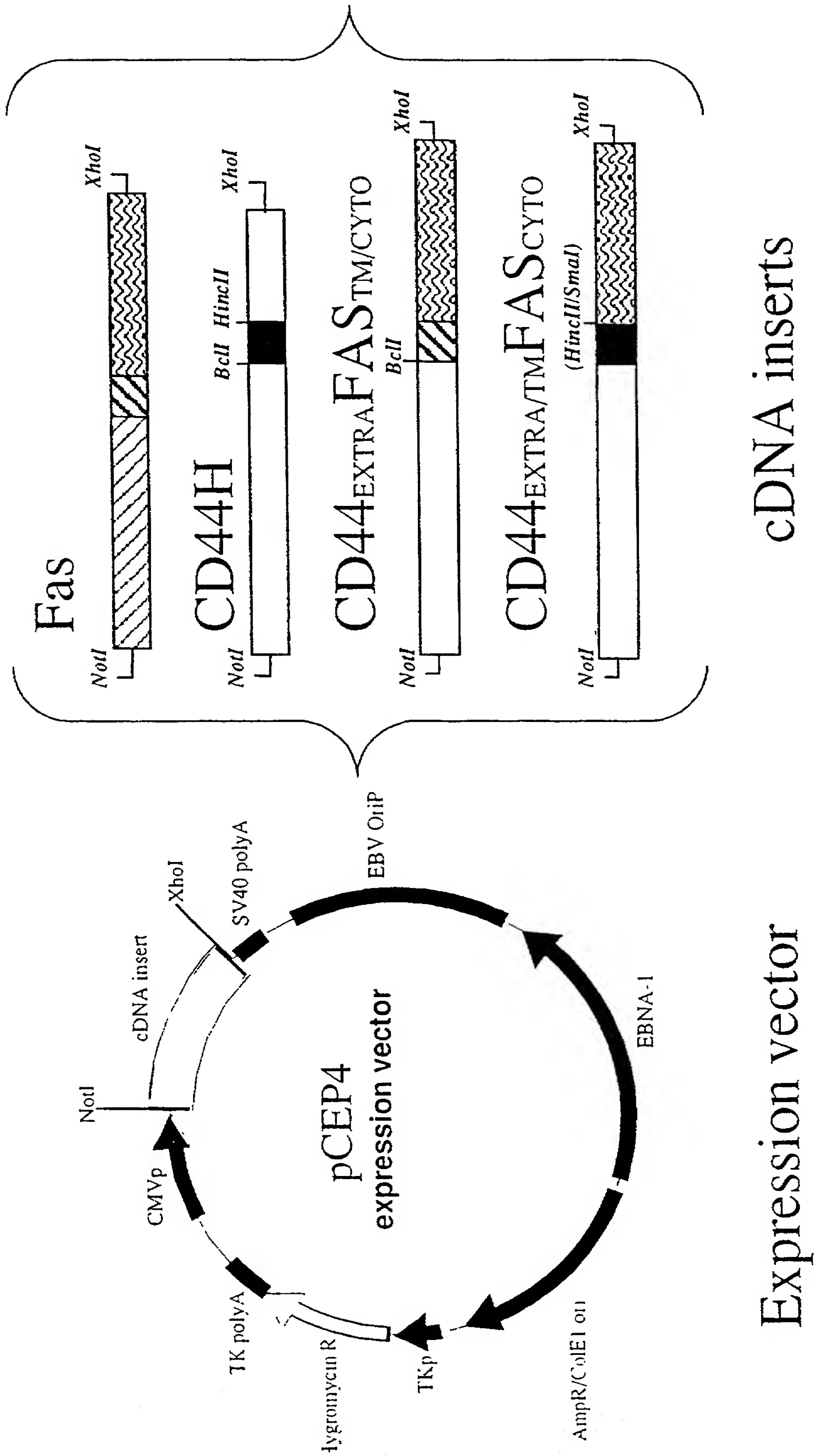
Fas
NotI
XhoI
CD44H
BclI
HincII
CD44EXTRAFASTM/CYTO
CD44EXTRA/TMFASCYTO
(HincII/SmaI)
cDNA inserts
pCEP4
expression vector
NotI
cDNA insert
XhoI
SV40 polyA
EBV OriP
EBNA-1
AmpR/ColE1 ori
TKp
hygromycin R
TK polyA
CMVp
Expression vector

FIGURE 1

CD44H^{EXTRA}FAS$^{TM/CYTO}$

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                                                                1
                                                                CCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCC
45
GCCGGCCCCTGCCCCGCGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACC ATG GAC
                                                                                    Met Asp
                                                                                    -19
122
AAG TTT TGG TGG CAC GCA GCC TGG GGA CTC TGC CTC GTG CCG CTG AGC CTG GCG CAG ATC
Lys Phe Trp Trp His Ala Ala Trp Gly Leu Cys Leu Val Pro Leu Ser Leu Ala Gln Ile
                                                                              +1
182
GAT TTG AAT ATA ACC TGC CGC TTT GCA GGT GTA TTC CAC GTG GAG AAA AAT GGT CGC TAC
Asp Leu Asn Ile Thr Cys Arg Phe Ala Gly Val Phe His Val Glu Lys Asn Gly Arg Tyr
                                                                              23
242
AGC ATC TCT CGG ACG GAG GCC GCT GAC CTC TGC AAG GCT TTC AAT AGC ACC TTG CCC ACA
Ser Ile Ser Arg Thr Glu Ala Ala Asp Leu Cys Lys Ala Phe Asn Ser Thr Leu Pro Thr
                                                                              43
302
ATG GCC CAG ATG GAG AAA GCT CTG AGC ATC GGA TTT GAG ACC TGC AGG TAT GGG TTC ATA
Met Ala Gln Met Glu Lys Ala Leu Ser Ile Gly Phe Glu Thr Cys Arg Tyr Gly Phe Ile
                                                                              63
362
GAA GGG CAT GTG GTG ATT CCC CGG ATC CAC CCC AAC TCC ATC TGT GCA GCA AAC AAC ACA
Glu Gly His Val Val Ile Pro Arg Ile His Pro Asn Ser Ile Cys Ala Ala Asn Asn Thr
                                                                              83
422
GGG GTG TAC ATC CTC ACA TAC AAC ACC TCC CAG TAT GAC ACA TAT TGC TTC AAT GCT TCA
Gly Val Tyr Ile Leu Thr Tyr Asn Thr Ser Gln Tyr Asp Thr Tyr Cys Phe Asn Ala Ser
                                                                             103
482
GCT CCA CCT GAA GAA GAT TGT ACA TCA GTC ACA GAC CTG CCC AAT GCC TTT GAT GGA CCA
Ala Pro Pro Glu Glu Asp Cys Thr Ser Val Thr Asp Leu Pro Asn Ala Phe Asp Gly Pro
                                                                             123
542
ATT ACC ATA ACT ATT GTT AAC CGT GAT GGC ACC CGC TAT GTC CAG AAA GGA GAA TAC AGA
Ile Thr Ile Thr Ile Val Asn Arg Asp Gly Thr Arg Tyr Val Gln Lys Gly Glu Tyr Arg
                                                                             143
602
ACG AAT CCT GAA GAC ATC TAC CCC AGC AAC CCT ACT GAT GAT GAC GTG AGC AGC GGC TCC
Thr Asn Pro Glu Asp Ile Tyr Pro Ser Asn Pro Thr Asp Asp Asp Val Ser Ser Gly Ser
                                                                             163
662
TCC AGT GAA AGG AGC AGC ACT TCA GGA GGT TAC ATC TTT TAC ACC TTT TCT ACT GTA CAC
Ser Ser Glu Arg Ser Ser Thr Ser Gly Gly Tyr Ile Phe Tyr Thr Phe Ser Thr Val His
                                                                             183
722
CCC ATC CCA GAC GAA GAC AGT CCC TGG ATC ACC GAC AGC ACA GAC AGA ATC CCT GCT ACC
Pro Ile Pro Asp Glu Asp Ser Pro Trp Ile Thr Asp Ser Thr Asp Arg Ile Pro Ala Thr
                                                                             203
782
AGA GAC CAA GAC ACA TTC CAC CCC AGT GGG GGG TCC CAT ACC ACT CAT GGA TCT GAA TCA
Arg Asp Gln Asp Thr Phe His Pro Ser Gly Gly Ser His Thr Thr His Gly Ser Glu Ser
                                                                             223
```

FIGURE 2A

```
842
GAT GGA CAC TCA CAT GGG AGT CAA GAA GGT GGA GCA AAC ACA ACC TCT GGT CCT ATA AGG
Asp Gly His Ser His Gly Ser Gln Glu Gly Gly Ala Asn Thr Thr Ser Gly Pro Ile Arg
                                                                          243
902                                  BclI
ACA CCC CAA ATT CCA GAA TGG CTG ATC ATC CTT TGT CTT CTT CTT TTG CCA ATT CCA CTA
Thr Pro Gln Ile Pro Glu Trp Leu Ile Ile Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu
                                                                          263
962
ATT GTT TGG GTG AAG AGA AAG GAA GTA CAG AAA ACA TGC AGA AAG CAC AGA AAG GAA AAC
Ile Val Trp Val Lys Arg Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn
                                                                          283
1022
CAA GGT TCT CAT GAA TCT CCA ACC TTA AAT CCT GAA ACA GTG GCA ATA AAT TTA TCT GAT
Gln Gly Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp
                                                                          303
1082
GTT GAC TTG AGT AAA TAT ATC ACC ACT ATT GCT GGA GTC ATG ACA CTA AGT CAA GTT AAA
Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val Lys
                                                                          323
1142
GGC TTT GTT CGA AAG AAT GGT GTC AAT GAA GCC AAA ATA GAT GAG ATC AAG AAT GAC AAT
Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn
                                                                          343
1202
GTC CAA GAC ACA GCA GAA CAG AAA GTT CAA CTG CTT CGT AAT TGG CAT CAA CTT CAT GGA
Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly
                                                                          363
1262
AAG AAA GAA GCG TAT GAC ACA TTG ATT AAA GAT CTC AAA AAA GCC AAT CTT TGT ACT CTT
Lys Lys Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu
                                                                          383
1322  #
GCA GGG AAA ATT CAG ACT ATC ATC CTC AAG GAC ATT ACT AGT GAC TCA GAA AAT TCA AAC
Ala Gly Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn
                                                                          403
1382
TTC AGA AAT GAA ATC CAA AGC TTG GTC TAG AGTGAAAAACAACAAATTCAGTTCTGAGTATATGCAATT
Phe Arg Asn Glu Ile Gln Ser Leu Val ***

1451
AGTGTTTGAAAAGATTCT
```

FIGURE 2B

$CD44H^{EXTRA/TM}FAS^{CYTO}$

```
                                                                1
                                                                CCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCC

45
GCCGGCCCCTGCCCCGCGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACC ATG GAC
                                                                          Met Asp
                                                                          -19

122
AAG TTT TGG TGG CAC GCA GCC TGG GGA CTC TGC CTC GTG CCG CTG AGC CTG GCG CAG ATC
Lys Phe Trp Trp His Ala Ala Trp Gly Leu Cys Leu Val Pro Leu Ser Leu Ala Gln Ile
                                                                          +1

182
GAT TTG AAT ATA ACC TGC CGC TTT GCA GGT GTA TTC CAC GTG GAG AAA AAT GGT CGC TAC
Asp Leu Asn Ile Thr Cys Arg Phe Ala Gly Val Phe His Val Glu Lys Asn Gly Arg Tyr
                                                                          23

242
AGC ATC TCT CGG ACG GAG GCC GCT GAC CTC TGC AAG GCT TTC AAT AGC ACC TTG CCC ACA
Ser Ile Ser Arg Thr Glu Ala Ala Asp Leu Cys Lys Ala Phe Asn Ser Thr Leu Pro Thr
                                                                          43

302
ATG GCC CAG ATG GAG AAA GCT CTG AGC ATC GGA TTT GAG ACC TGC AGG TAT GGG TTC ATA
Met Ala Gln Met Glu Lys Ala Leu Ser Ile Gly Phe Glu Thr Cys Arg Tyr Gly Phe Ile
                                                                          63

362
GAA GGG CAT GTG GTG ATT CCC CGG ATC CAC CCC AAC TCC ATC TGT GCA GCA AAC AAC ACA
Glu Gly His Val Val Ile Pro Arg Ile His Pro Asn Ser Ile Cys Ala Ala Asn Asn Thr
                                                                          83

422
GGG GTG TAC ATC CTC ACA TAC AAC ACC TCC CAG TAT GAC ACA TAT TGC TTC AAT GCT TCA
Gly Val Tyr Ile Leu Thr Tyr Asn Thr Ser Gln Tyr Asp Thr Tyr Cys Phe Asn Ala Ser
                                                                          103

482
GCT CCA CCT GAA GAA GAT TGT ACA TCA GTC ACA GAC CTG CCC AAT GCC TTT GAT GGA CCA
Ala Pro Pro Glu Glu Asp Cys Thr Ser Val Thr Asp Leu Pro Asn Ala Phe Asp Gly Pro
                                                                          123

542
ATT ACC ATA ACT ATT GTT AAC CGT GAT GGC ACC CGC TAT GTC CAG AAA GGA GAA TAC AGA
Ile Thr Ile Thr Ile Val Asn Arg Asp Gly Thr Arg Tyr Val Gln Lys Gly Glu Tyr Arg
                                                                          143

602
ACG AAT CCT GAA GAC ATC TAC CCC AGC AAC CCT ACT GAT GAT GAC GTG AGC AGC GGC TCC
Thr Asn Pro Glu Asp Ile Tyr Pro Ser Asn Pro Thr Asp Asp Asp Val Ser Ser Gly Ser
                                                                          163

662
TCC AGT GAA AGG AGC AGC ACT TCA GGA GGT TAC ATC TTT TAC ACC TTT TCT ACT GTA CAC
Ser Ser Glu Arg Ser Ser Thr Ser Gly Gly Tyr Ile Phe Tyr Thr Phe Ser Thr Val His
                                                                          183

722
CCC ATC CCA GAC GAA GAC AGT CCC TGG ATC ACC GAC AGC ACA GAC AGA ATC CCT GCT ACC
Pro Ile Pro Asp Glu Asp Ser Pro Trp Ile Thr Asp Ser Thr Asp Arg Ile Pro Ala Thr
                                                                          203

782
AGA GAC CAA GAC ACA TTC CAC CCC AGT GGG GGG TCC CAT ACC ACT CAT GGA TCT GAA TCA
Arg Asp Gln Asp Thr Phe His Pro Ser Gly Gly Ser His Thr Thr His Gly Ser Glu Ser
                                                                          223
```

FIGURE 3A

842
GAT GGA CAC TCA CAT GGG AGT CAA GAA GGT GGA GCA AAC ACA ACC TCT GGT CCT ATA AGG
Asp Gly His Ser His Gly Ser Gln Glu Gly Gly Ala Asn Thr Thr Ser Gly Pro Ile Arg
243

902
ACA CCC CAA ATT CCA GAA TGG CTG ATC ATC TTG GCA TCC CTC TTG GCC TTG GCT TTG ATT
Thr Pro Gln Ile Pro Glu **Trp Leu Ile Ile Leu Ala Ser Leu Leu Ala Leu Ala Leu Ile**
263

962 junction
CTT GCA GTT TGC ATT GCA GTC *GGG GTG AAG AGA AAG GAA GTA CAG AAA ACA TGC AGA AAG*
Leu Ala Val Cys Ile Ala Val Gly *Val Lys Arg Lys Glu Val Gln Lys Thr Cys Arg Lys*
283

1022
CAC AGA AAG GAA AAC CAA GGT TCT CAT GAA TCT CCA ACC TTA AAT CCT GAA ACA GTG GCA
His Arg Lys Glu Asn Gln Gly Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala
303

1082
ATA AAT TTA TCT GAT GTT GAC TTG AGT AAA TAT ATC ACC ACT ATT GCT GGA GTC ATG ACA
Ile Asn Leu Ser Asp Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr
323

1142
CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT GGT GTC AAT GAA GCC AAA ATA GAT GAG
Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile Asp Glu
343

1202
ATC AAG AAT GAC AAT GTC CAA GAC ACA GCA GAA CAG AAA GTT CAA CTG CTT CGT AAT TGG
Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp
363

1262
CAT CAA CTT CAT GGA AAG AAA GAA GCG TAT GAC ACA TTG ATT AAA GAT CTC AAA AAA GCC
His Gln Leu His Gly Lys Lys Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala
383

1322 #
AAT CTT TGT ACT CTT GCA GGG AAA ATT CAG ACT ATC ATC CTC AAG GAC ATT ACT AGT GAC
Asn Leu Cys Thr Leu Ala Gly Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp
403

1382
TCA GAA AAT TCA AAC TTC AGA AAT GAA ATC CAA AGC TTG GTC TAG AGTGAAAAACAACAAATTC
Ser Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln Ser Leu Val ***

1442
AGTTCTGAGTATATGCAATTAGTGTTTGAAAAGATTCT

FIGURE 3B

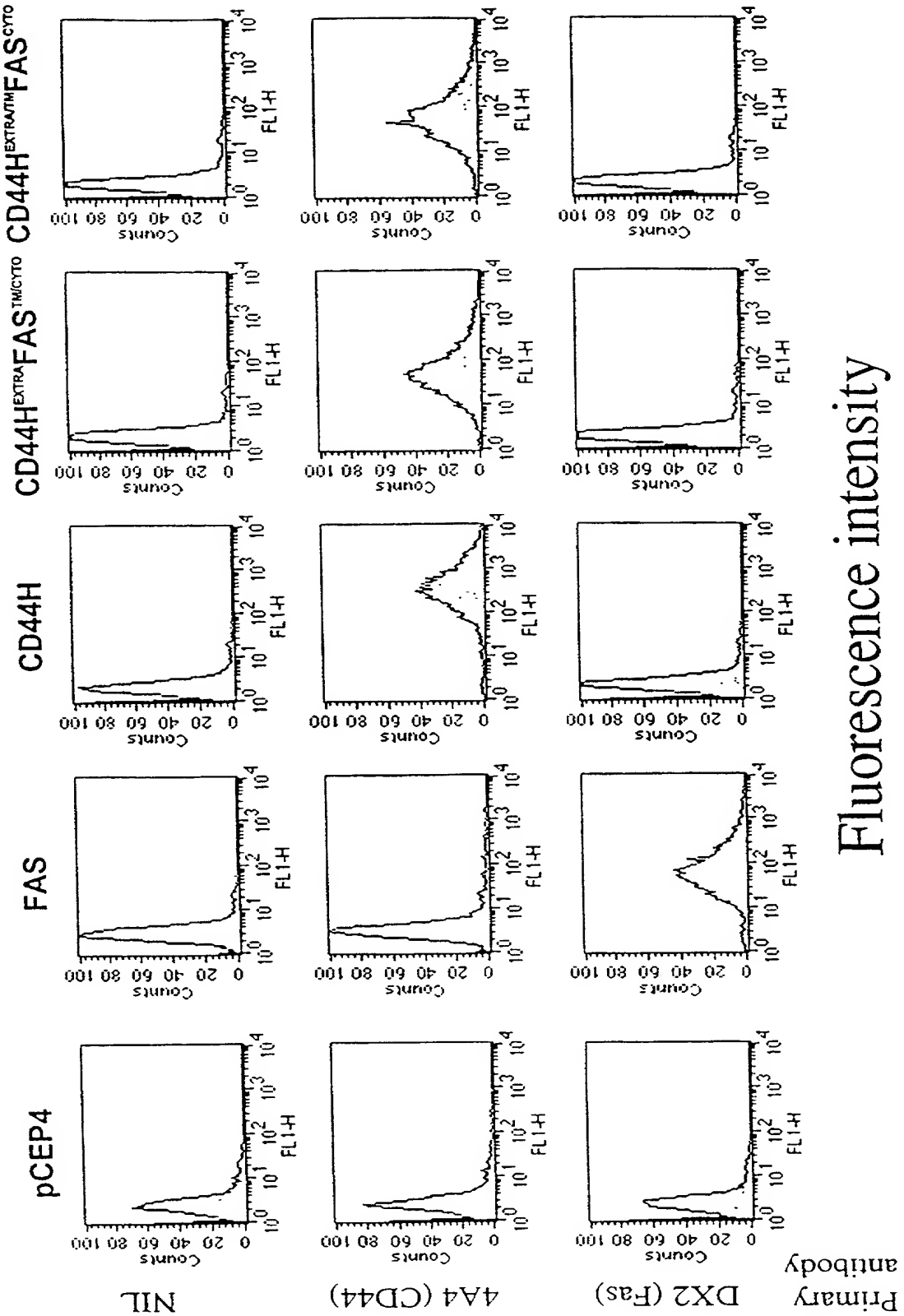
Primary antibody
DX2 (Fas)
4A4 (CD44)
NIL
Cell number
Fluorescence intensity
pCEP4
FAS
CD44H
CD44H^EXTRA FAS^TM/CYTO
CD44H^EXTRA/TM FAS^CYTO
Counts
FL1-H

FIGURE 4

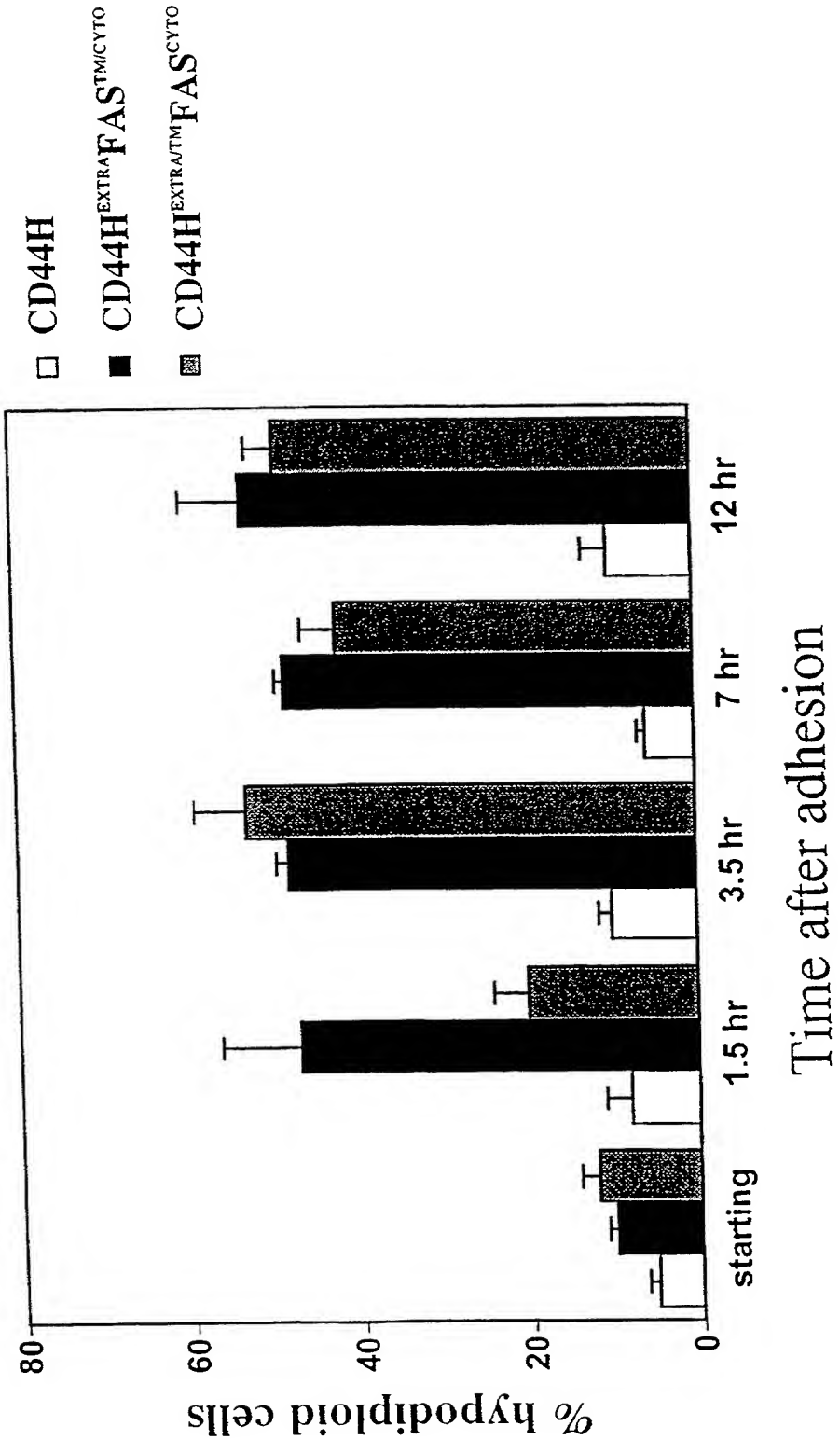
CD44H
CD44H^EXTRA FAS^TM/CYTO
CD44H^EXTRA/TM FAS^CYTO
% hypodiploid cells
0
20
40
60
80
starting
1.5 hr
3.5 hr
7 hr
12 hr
Time after adhesion

FIGURE 5

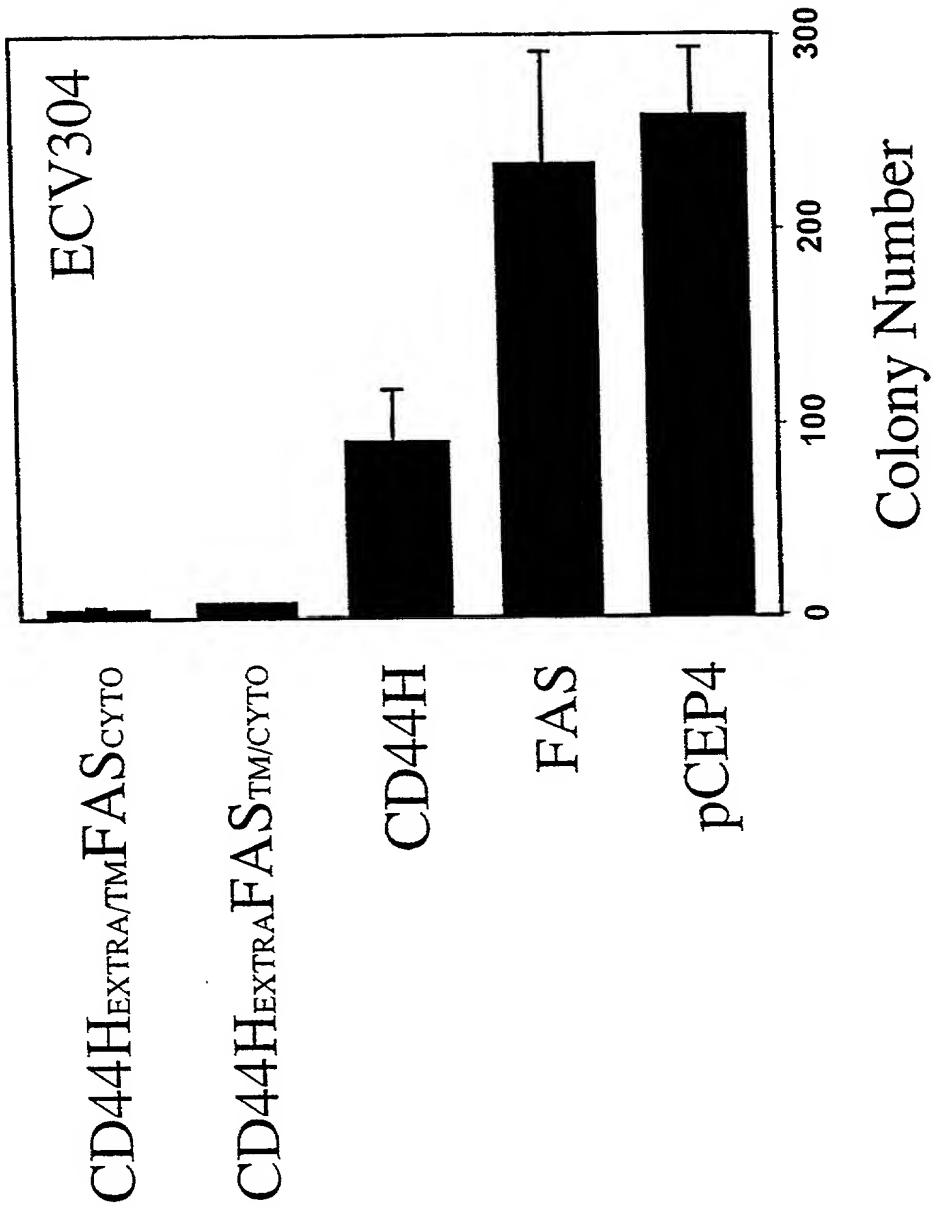
ECV304
0
100
200
300
Colony Number
CD44HEXTRA/TMFASCYTO
CD44HEXTRAFASTM/CYTO
CD44H
FAS
pCEP4

FIGURE 6

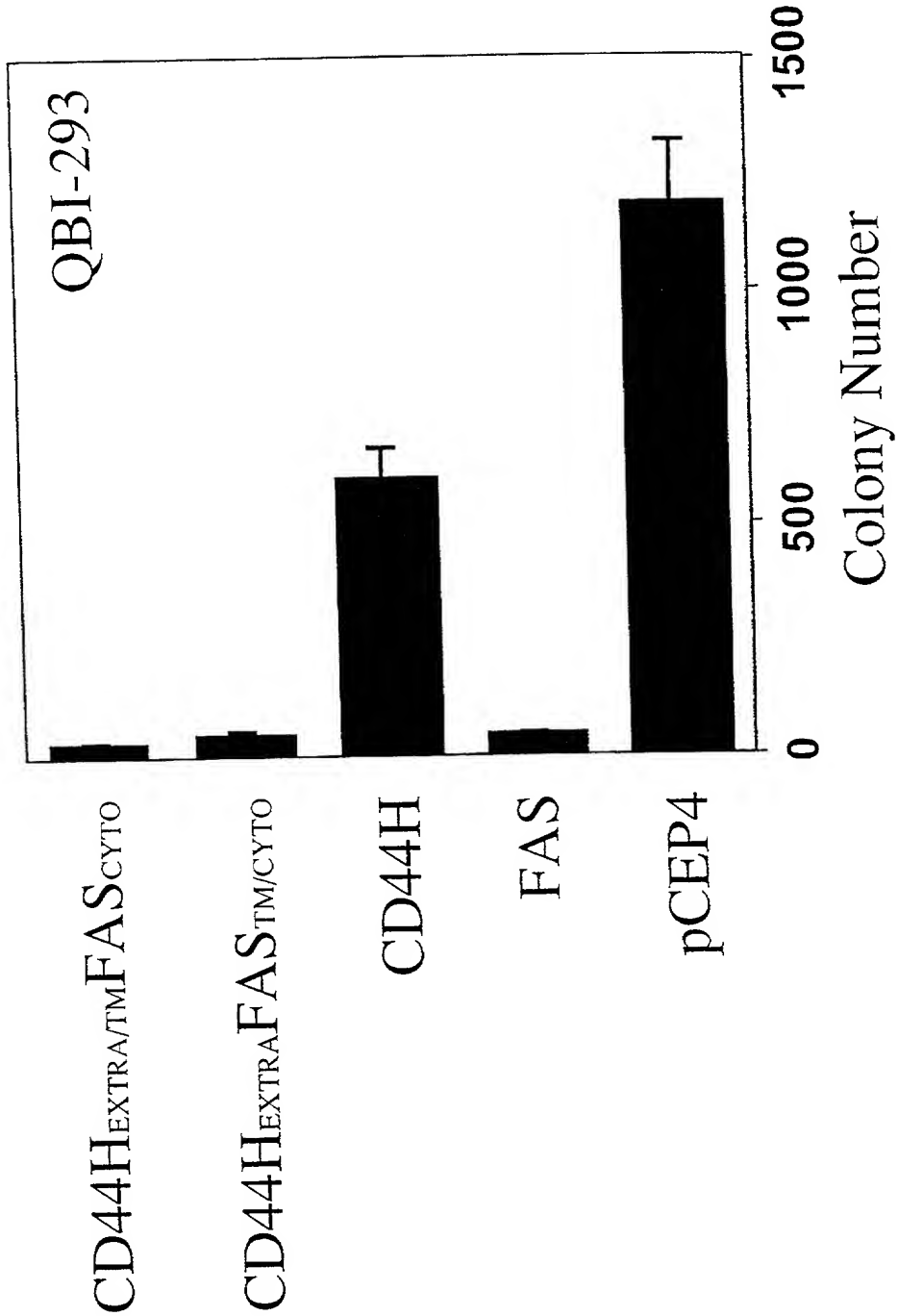

FIGURE 7

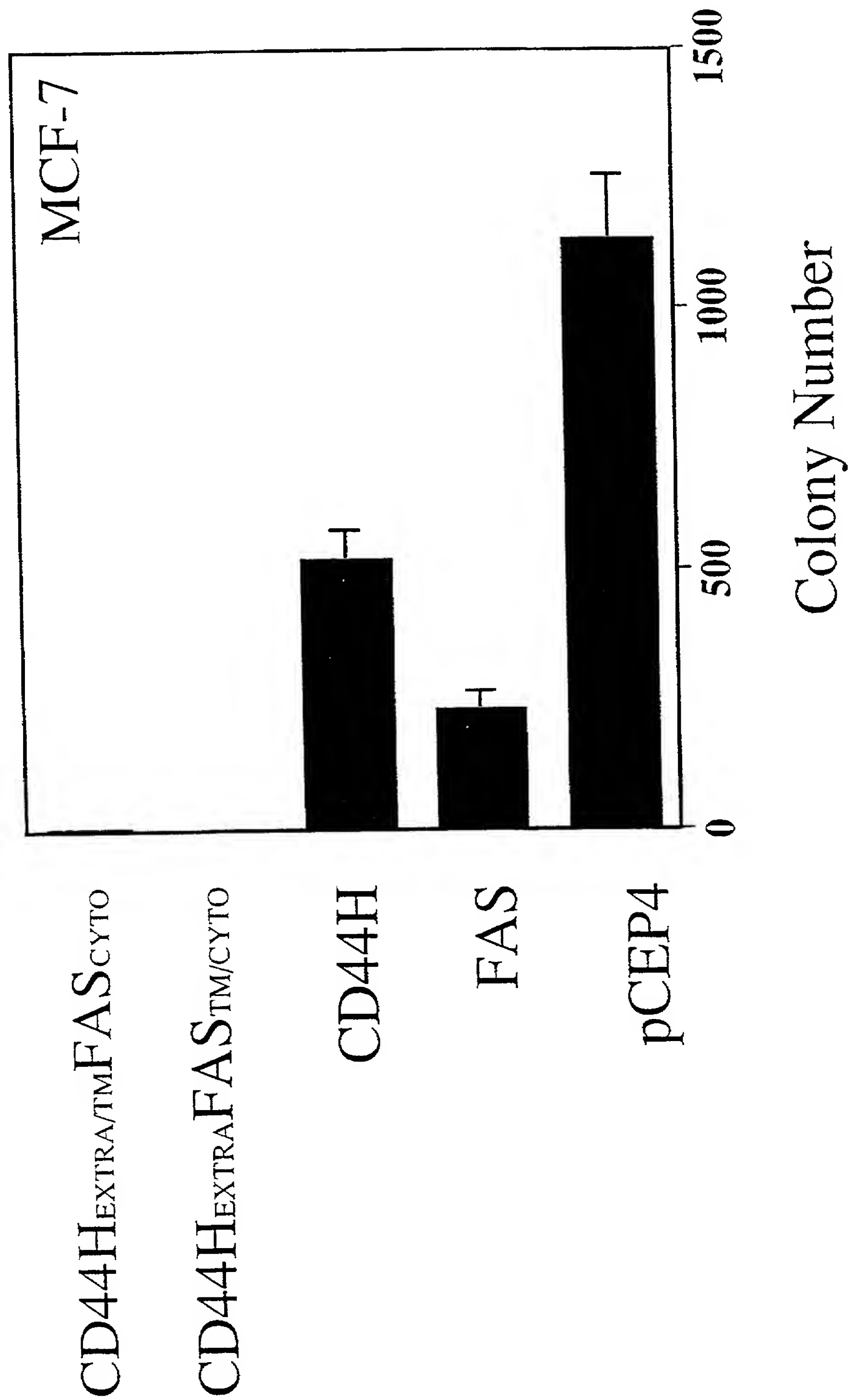
MCF-7
CD44H$_{EXTRA/TM}$FAS$_{CYTO}$
CD44H$_{EXTRA}$FAS$_{TM/CYTO}$
CD44H
FAS
pCEP4
0
500
1000
1500
Colony Number

FIGURE 8

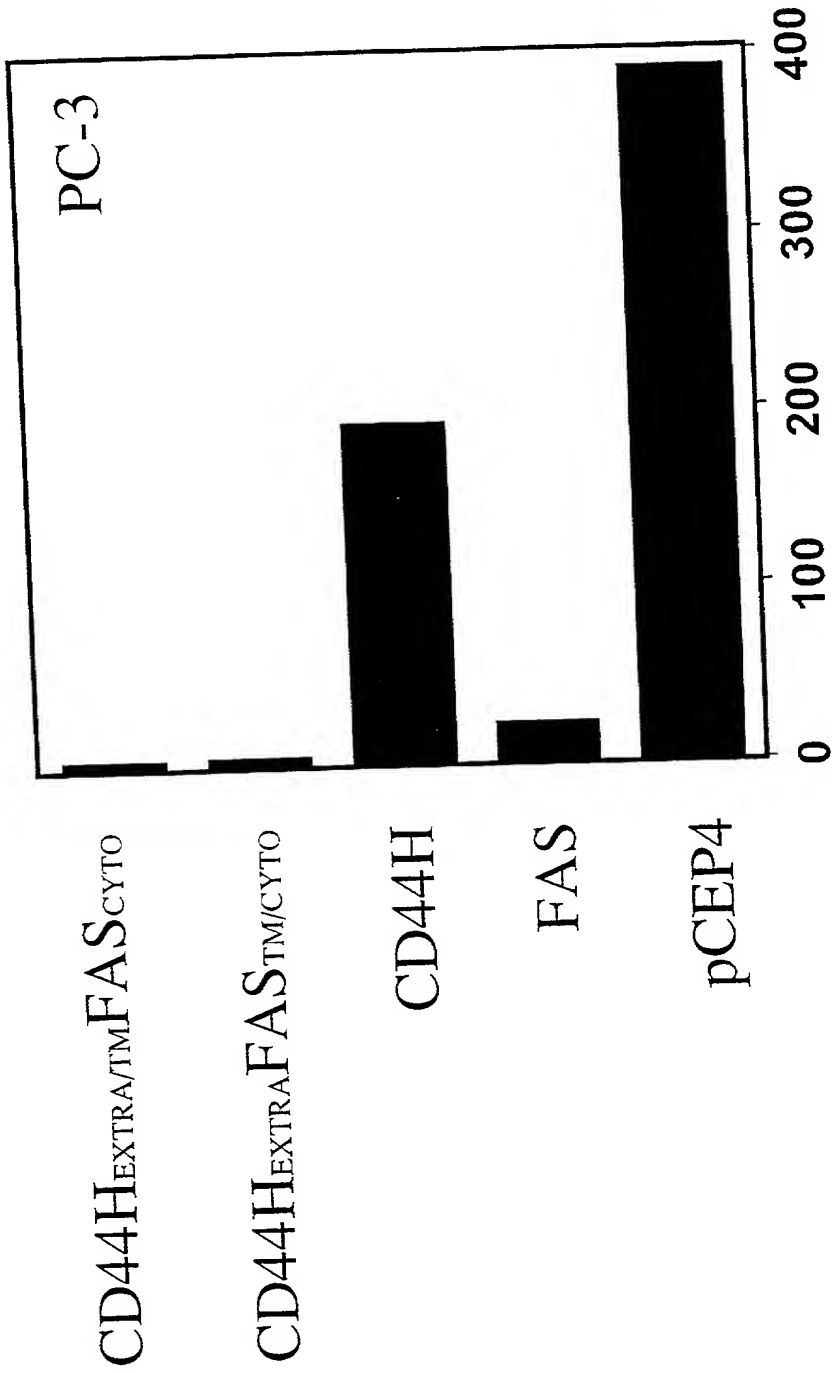
PC-3
0
100
200
300
400
Colony Number
CD44HEXTRA/TMFASCYTO
CD44HEXTRAFASTM/CYTO
CD44H
FAS
pCEP4

FIGURE 9

Flt-1^{EXTRA}Fas$^{TM/CYTO}$

```
                                                                  1
                                                                  GCGGGTACCGCGGCCAGCGG
GCCTGGCGGCCGAGGATTACCCGGGGAAGTGGTTGTCTCCTGGCTGGAGCCGCGAGACGGGCGCTCAGGGCGCGGGGCCG
GCGGCCGGCGAACGACAGCACGGACTCTGGCGGCCGGGTCGTTGGCCGGGGGAGCGCGGGCACCGGGCGAGCAGGCCGCG

TCGCGCTCACC ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT
            Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys
            -22
241
CTG CTT CTC ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA
Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys
                   +1                                                        15
301
GGC ACC CAG CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA
Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala
                                                                          35
361
GCC CAT AAA TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT
Ala His Lys Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr
                                                                          55
421
AAA TCT GCC TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT
Lys Ser Ala Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala
                                                                          75
481
CAA GCA AAC CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG
Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys
                                                                          95
541
AAG GAA ACA GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG
Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu
                                                                         115
601
ATG TAC AGT GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro
                                                                         135
661
TGC CGG GTT ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG
Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu
                                                                         155
721
ATC CCT GAT GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA
Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala
                                                                         175
781
ACG TAC AAA GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG
Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
                                                                         195
841
ACA AAC TAT CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro
                                                                         215
901
CGC CCA GTC AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC
Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro
```


FIGURE 10A

```
                                                                    235
961
TTG AAC ACG AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC
Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser
                                                                    255
1021
GTA AGG CGA CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT
Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr
                                                                    275
1081
ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA
Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro
                                                                    295
1141
TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA TTC ATC ACT GTG AAA
Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Phe Ile Thr Val Lys
                                                                    315
1201
CAT CGA AAA CAG CAG GTG CTT GAA ACC GTA GCT GGC AAG CGG TCT TAC CGG CTC TCT ATG
His Arg Lys Gln Gln Val Leu Glu Thr Val Ala Gly Lys Arg Ser Tyr Arg Leu Ser Met
                                                                    335
1261
AAA GTG AAG GCA TTT CCC TCG CCG GAA GTT GTA TGG TTA AAA GAT GGG TTA CCT GCG ACT
Lys Val Lys Ala Phe Pro Ser Pro Glu Val Val Trp Leu Lys Asp Gly Leu Pro Ala Thr
                                                                    335
1321
GAG AAA TCT GCT CGC TAT TTG ACT CGT GGC TAC TCG TTA ATT ATC AAG GAC GTA ACT GAA
Glu Lys Ser Ala Arg Tyr Leu Thr Arg Gly Tyr Ser Leu Ile Ile Lys Asp Val Thr Glu
                                                                    355
1381
GAG GAT GCA GGG AAT TAT ACA ATC TTG CTG AGC ATA AAA CAG TCA AAT GTG TTT AAA AAC
Glu Asp Ala Gly Asn Tyr Thr Ile Leu Leu Ser Ile Lys Gln Ser Asn Val Phe Lys Asn
                                                                    375
1441
CTC ACT GCC ACT CTA ATT GTC AAT GTG AAA CCC CAG ATT TAC GAA AAG GCC GTG TCA TCG
Leu Thr Ala Thr Leu Ile Val Asn Val Lys Pro Gln Ile Tyr Glu Lys Ala Val Ser Ser
                                                                    395
1501
TTT CCA GAC CCG GCT CTC TAC CCA CTG GGC AGC AGA CAA ATC CTG ACT TGT ACC GCA TAT
Phe Pro Asp Pro Ala Leu Tyr Pro Leu Gly Ser Arg Gln Ile Leu Thr Cys Thr Ala Tyr
                                                                    415
1561
GGT ATC CCT CAA CCT ACA ATC AAG TGG TTC TGG CAC CCC TGT AAC CAT AAT CAT TCC GAA
Gly Ile Pro Gln Pro Thr Ile Lys Trp Phe Trp His Pro Cys Asn His Asn His Ser Glu
                                                                    435
1621
GCA AGG TGT GAC TTT TGT TCC AAT AAT GAA GAG TCC TTT ATC CTG GAT GCT GAC AGC AAC
Ala Arg Cys Asp Phe Cys Ser Asn Asn Glu Glu Ser Phe Ile Leu Asp Ala Asp Ser Asn
                                                                    455
1681
ATG GGA AAC AGA ATT GAG AGC ATC ACT CAG CGC ATG GCA ATA ATA GAA GGA AAG AAT AAG
Met Gly Asn Arg Ile Glu Ser Ile Thr Gln Arg Met Ala Ile Ile Glu Gly Lys Asn Lys
                                                                    475
1741
ATG GCT AGC ACC TTG GTT GTG GCT GAC TCT AGA ATT TCT GGA ATC TAC ATT TGC ATA GCT
Met Ala Ser Thr Leu Val Val Ala Asp Ser Arg Ile Ser Gly Ile Tyr Ile Cys Ile Ala
                                                                    495
```

FIGURE 10B

```
1801
TCC AAT AAA GTT GGG ACT GTG GGA AGA AAC ATA AGC TTT TAT ATC ACA GAT GTG CCA AAT
Ser Asn Lys Val Gly Thr Val Gly Arg Asn Ile Ser Phe Tyr Ile Thr Asp Val Pro Asn
                                                                          515
1861
GGG TTT CAT GTT AAC TTG GAA AAA ATG CCG ACG GAA GGA GAG GAC CTG AAA CTG TCT TGC
Gly Phe His Val Asn Leu Glu Lys Met Pro Thr Glu Gly Glu Asp Leu Lys Leu Ser Cys
                                                                          535
1921
ACA GTT AAC AAG TTC TTA TAC AGA GAC GTT ACT TGG ATT TTA CTG CGG ACA GTT AAT AAC
Thr Val Asn Lys Phe Leu Tyr Arg Asp Val Thr Trp Ile Leu Leu Arg Thr Val Asn Asn
                                                                          555
1981
AGA ACA ATG CAC TAC AGT ATT AGC AAG CAA AAA ATG GCC ATC ACT AAG GAG CAC TCC ATC
Arg Thr Met His Tyr Ser Ile Ser Lys Gln Lys Met Ala Ile Thr Lys Glu His Ser Ile
                                                                          575
2041
ACT CTT AAT CTT ACC ATC ATG AAT GTT TCC CTG CAA GAT TCA GGC ACC TAT GCC TGC AGA
Thr Leu Asn Leu Thr Ile Met Asn Val Ser Leu Gln Asp Ser Gly Thr Tyr Ala Cys Arg
                                                                          595
2101
GCC AGG AAT GTA TAC ACA GGG GAA GAA ATC CTC CAG AAG AAA GAA ATT ACA ATC AGA GAT
Ala Arg Asn Val Tyr Thr Gly Glu Glu Ile Leu Gln Lys Lys Glu Ile Thr Ile Arg Asp
                                                                          615
2161
CAG GAA GCA CCA TAC CTC CTG CGA AAC CTC AGT GAT CAC ACA GTG GCC ATC AGC AGT TCC
Gln Glu Ala Pro Tyr Leu Leu Arg Asn Leu Ser Asp His Thr Val Ala Ile Ser Ser Ser
                                                                          635
2221
ACC ACT TTA GAC TGT CAT GCT AAT GGT GTC CCC GAG CCT CAG ATC ACT TGG TTT AAA AAC
Thr Thr Leu Asp Cys His Ala Asn Gly Val Pro Glu Pro Gln Ile Thr Trp Phe Lys Asn
                                                                          655
2281
AAC CAC AAA ATA CAA CAA GAG CCT GGA ATT ATT TTA GGA CCA GGA AGC AGC ACG CTG TTT
Asn His Lys Ile Gln Gln Glu Pro Gly Ile Ile Leu Gly Pro Gly Ser Ser Thr Leu Phe
                                                                          675
2341
ATT GAA AGA GTC ACA GAA GAG GAT GAA GGT GTC TAT CAC TGC AAA GCC ACC AAC CAG AAG
Ile Glu Arg Val Thr Glu Glu Asp Glu Gly Val Tyr His Cys Lys Ala Thr Asn Gln Lys
                                                                          695
2401                                                          BamHI
GGC TCT GTG GAA AGT TCA GCA TAC CTC ACT GTT CAA GGA ACC TCG GAC GGA TCC AGA TCT
Gly Ser Val Glu Ser Ser Ala Tyr Leu Thr Val Gln Gly Thr Ser Asp Gly Ser Arg Ser
                                                                          715
2461
AAC TTG GGG TGG CTT TGT CTT CTT CTT TTG CCA ATT CCA CTA ATT GTT TGG GTG AAG AGA
Asn Leu Gly Trp Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
                                                                          735
2521
AAG GAA GTA CAG AAA ACA TGC AGA AAG CAC AGA AAG GAA AAC CAA GGT TCT CAT GAA TCT
Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly Ser His Glu Ser
                                                                          755
2581
CCA ACC TTA AAT CCT GAA ACA GTG GCA ATA AAT TTA TCT GAT GTT GAC TTG AGT AAA TAT
Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp Val Asp Leu Ser Lys Tyr
                                                                          775
```

FIGURE 10C

```
2641
ATC ACC ACT ATT GCT GGA GTC ATG ACA CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT
Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn
                                                                          795
2701
GGT GTC AAT GAA GCC AAA ATA GAT GAG ATC AAG AAT GAC AAT GTC CAA GAC ACA GCA GAA
Gly Val Asn Glu Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu
                                                                          815
2761
CAG AAA GTT CAA CTG CTT CGT AAT TGG CAT CAA CTT CAT GGA AAG AAA GAA GCG TAT GAC
Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala Tyr Asp
                                                                          835
2821                                                    #
ACA TTG ATT AAA GAT CTC AAA AAA GCC AAT CTT TGT ACT CTT GCA GGG AAA ATT CAG ACT
Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala Gly Lys Ile Gln Thr
                                                                          855
2881
ATC ATC CTC AAG GAC ATT ACT AGT GAC TCA GAA AAT TCA AAC TTC AGA AAT GAA ATC CAA
Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln
                                                                          875
2941
AGC TTG GTC TAG AGTGAAAAACAACAAATTCAGTTCTGAGTATATGCAATTAGTGTTTGAAAAGATTCT
Ser Leu Val ***
```

Flk-1^{EXTRA}Fas$^{TM/CYTO}$

```
                                                                1
                                                                GCGGGTACCGCCGCCCGGTCGCGCGCCC
GGGCTCCCTAGCCCTGTGCGCTCAACTGTCCTGCGCTGCGGGGTGCCGCGAGTTCCACCTCCGCGCCTCCTTCTCTAGA

CAGCGCCTGGGAGAAAGAACCGGCTCCCGAGTTCTGGGCATTTCGCCCGGCTCGAGGTGCAGG ATG CAG AGC AAG
                                                                  Met Gln Ser Lys
                                                               -19
181
GTG CTG CTG GCC GTC GCC CTG TGG CTC TGC GTG GAG ACC CGG GCC GCC TCT GTG GGT TTG
Val Leu Leu Ala Val Ala Leu Trp Leu Cys Val Glu Thr Arg Ala Ala Ser Val Gly Leu
                                                            +1
241
CCT AGT GTT TCT CTT GAT CTG CCC AGG CTC AGC ATA CAA AAA GAC ATA CTT ACA ATT AAG
Pro Ser Val Ser Leu Asp Leu Pro Arg Leu Ser Ile Gln Lys Asp Ile Leu Thr Ile Lys
                                                                              25
301
GCT AAT ACA ACT CTT CAA ATT ACT TGC AGG GGA CAG AGG GAC TTG GAC TGG CTT TGG CCC
Ala Asn Thr Thr Leu Gln Ile Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro
                                                                              45
361
AAT AAT CAG AGT GGC AGT GAG CAA AGG GTG GAG GTG ACT GAG TGC AGC GAT GGC CTC TTC
Asn Asn Gln Ser Gly Ser Glu Gln Arg Val Glu Val Thr Glu Cys Ser Asp Gly Leu Phe
                                                                              65
421
TGT AAG ACA CTC ACA ATT CCA AAA GTG ATC GGA AAT GAC ACT GGA GCC TAC AAG TGC TTC
Cys Lys Thr Leu Thr Ile Pro Lys Val Ile Gly Asn Asp Thr Gly Ala Tyr Lys Cys Phe
                                                                              85
481
TAC CGG GAA ACT GAC TTG GCC TCG GTC ATT TAT GTC TAT GTT CAA GAT TAC AGA TCT CCA
Tyr Arg Glu Thr Asp Leu Ala Ser Val Ile Tyr Val Tyr Val Gln Asp Tyr Arg Ser Pro
                                                                             105
541
TTT ATT GCT TCT GTT AGT GAC CAA CAT GGA GTC GTG TAC ATT ACT GAG AAC AAA AAC AAA
Phe Ile Ala Ser Val Ser Asp Gln His Gly Val Val Tyr Ile Thr Glu Asn Lys Asn Lys
                                                                             125
601
ACT GTG GTG ATT CCA TGT CTC GGG TCC ATT TCA AAT CTC AAC GTG TCA CTT TGT GCA AGA
Thr Val Val Ile Pro Cys Leu Gly Ser Ile Ser Asn Leu Asn Val Ser Leu Cys Ala Arg
                                                                             145
661
TAC CCA GAA AAG AGA TTT GTT CCT GAT GGT AAC AGA ATT TCC TGG GAC AGC AAG AAG GGC
Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg Ile Ser Trp Asp Ser Lys Lys Gly
                                                                             165
721
TTT ACT ATT CCC AGC TAC ATG ATC AGC TAT GCT GGC ATG GTC TTC TGT GAA GCA AAA ATT
Phe Thr Ile Pro Ser Tyr Met Ile Ser Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile
                                                                             185
781
AAT GAT GAA AGT TAC CAG TCT ATT ATG TAC ATA GTT GTC GTT GTA GGG TAT AGG ATT TAT
Asn Asp Glu Ser Tyr Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg Ile Tyr
                                                                             205
841
GAT GTG GTT CTG AGT CCG TCT CAT GGA ATT GAA CTA TCT GTT GGA GAA AAG CTT GTC TTA
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val Leu
                                                                             225
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FIGURE 11A

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901
AAT TGT ACA GCA AGA ACT GAA CTA AAT GTG GGG ATT GAC TTC AAC TGG GAA TAC CCT TCT
Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser
                                                                          245
961
TCG AAG CAT CAG CAT AAG AAA CTT GTA AAC CGA GAC CTA AAA ACC CAG TCT GGG AGT GAG
Ser Lys His Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu
                                                                          265
1021
ATG AAG AAA TTT TTG AGC ACC TTA ACT ATA GAT GGT GTA ACC CGG AGT GAC CAA GGA TTG
Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
                                                                          285
1081
TAC ACC TGT GCA GCA TCC AGT GGG CTG ATG ACC AAG AAG AAC AGC ACA TTT GTC AGG GTC
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg Val
                                                                          305
1141
CAT GAA AAA CCT TTT GTT GCT TTT GGA AGT GGC ATG GAA TCT CTG GTG GAA GCC ACG GTG
His Glu Lys Pro Phe Val Ala Phe Gly Ser Gly Met Glu Ser Leu Val Glu Ala Thr Val
                                                                          325
1201
GGG GAG CGT GTC AGA ATC CCT GCG AAG TAC CTT GGT TAC CCA CCC CCA GAA ATA AAA TGG
Gly Glu Arg Val Arg Ile Pro Ala Lys Tyr Leu Gly Tyr Pro Pro Pro Glu Ile Lys Trp
                                                                          345
1261
TAT AAA AAT GGA ATA CCC CTT GAG TCC AAT CAC ACA ATT AAA GCG GGG CAT GTA CTG ACG
Tyr Lys Asn Gly Ile Pro Leu Glu Ser Asn His Thr Ile Lys Ala Gly His Val Leu Thr
                                                                          365
1321
ATT ATG GAA GTG AGT GAA AGA GAC ACA GGA AAT TAC ACT GTC ATC CTT ACC AAT CCC ATT
Ile Met Glu Val Ser Glu Arg Asp Thr Gly Asn Tyr Thr Val Ile Leu Thr Asn Pro Ile
                                                                          385
1381
TCA AAG GAG AAG CAG AGC CAT GTG GTC TCT CTG GTT GTG TAT GTC CCA CCC CAG ATT GGT
Ser Lys Glu Lys Gln Ser His Val Val Ser Leu Val Val Tyr Val Pro Pro Gln Ile Gly
                                                                          405
1441
GAG AAA TCT CTA ATC TCT CCT GTG GAT TCC TAC CAG TAC GGC ACC ACT CAA ACG CTG ACA
Glu Lys Ser Leu Ile Ser Pro Val Asp Ser Tyr Gln Tyr Gly Thr Thr Gln Thr Leu Thr
                                                                          425
1501
TGT ACG GTC TAT GCC ATT CCT CCC CCG CAT CAC ATC CAC TGG TAT TGG CAG TTG GAG GAA
Cys Thr Val Tyr Ala Ile Pro Pro Pro His His Ile His Trp Tyr Trp Gln Leu Glu Glu
                                                                          445
1561
GAG TGC GCC AAC GAG CCC AGC CAA GCT GTC TCA GTG ACA AAC CCA TAC CCT TGT GAA GAA
Glu Cys Ala Asn Glu Pro Ser Gln Ala Val Ser Val Thr Asn Pro Tyr Pro Cys Glu Glu
                                                                          465
1621
TGG AGA AGT GTG GAG GAC TTC CAG GGA GGA AAT AAA ATT GAA GTT AAT AAA AAT CAA TTT
Trp Arg Ser Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu Val Asn Lys Asn Gln Phe
                                                                          485
1681
GCT CTA ATT GAA GGA AAA AAC AAA ACT GTA AGT ACC CTT GTT ATC CAA GCG GCA AAT GTG
Ala Leu Ile Glu Gly Lys Asn Lys Thr Val Ser Thr Leu Val Ile Gln Ala Ala Asn Val
                                                                          505
```

FIGURE 11B

1741
TCA GCT TTG TAC AAA TGT GAA GCG GTC AAC AAA GTC GGG AGA GGA GAG AGG GTG ATC TCC
Ser Ala Leu Tyr Lys Cys Glu Ala Val Asn Lys Val Gly Arg Gly Glu Arg Val Ile Ser
525

1801
TTC CAC GTG ACC AGG GGT CCT GAA ATT ACT TTG CAA CCT GAC ATG CAG CCC ACT GAG CAG
Phe His Val Thr Arg Gly Pro Glu Ile Thr Leu Gln Pro Asp Met Gln Pro Thr Glu Gln
545

1861
GAG AGC GTG TCT TTG TGG TGC ACT GCA GAC AGA TCT ACG TTT GAG AAC CTC ACA TGG TAC
Glu Ser Val Ser Leu Trp Cys Thr Ala Asp Arg Ser Thr Phe Glu Asn Leu Thr Trp Tyr
565

1921
AAG CTT GGC CCA CAG CCT CTG CCA ATC CAT GTG GGA GAG TTG CCC ACA CCT GTT TGC AAG
Lys Leu Gly Pro Gln Pro Leu Pro Ile His Val Gly Glu Leu Pro Thr Pro Val Cys Lys
585

1981
AAC TTG GAT ACT CTT TGG AAA TTG AAT GCC ACC ATG TTC TCT AAT AGC ACA AAT GAC ATT
Asn Leu Asp Thr Leu Trp Lys Leu Asn Ala Thr Met Phe Ser Asn Ser Thr Asn Asp Ile
605

2041
TTG ATC ATG GAG CTT AAG AAT GCA TCC TTG CAG GAC CAA GGA GAC TAT GTC TGC CTT GCT
Leu Ile Met Glu Leu Lys Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys Leu Ala
625

2101
CAA GAC AGG AAG ACC AAG AAA AGA CAT TGC GTG GTC AGG CAG CTC ACA GTC CTA GAG CGT
Gln Asp Arg Lys Thr Lys Lys Arg His Cys Val Val Arg Gln Leu Thr Val Leu Glu Arg
645

2161
GTG GCA CCC ACG ATC ACA GGA AAC CTG GAG AAT CAG ACG ACA AGT ATT GGG GAA AGC ATC
Val Ala Pro Thr Ile Thr Gly Asn Leu Glu Asn Gln Thr Thr Ser Ile Gly Glu Ser Ile
665

2221
GAA GTC TCA TGC ACG GCA TCT GGG AAT CCC CCT CCA CAG ATC ATG TGG TTT AAA GAT AAT
Glu Val Ser Cys Thr Ala Ser Gly Asn Pro Pro Pro Gln Ile Met Trp Phe Lys Asp Asn
685

2281
GAG ACC CTT GTA GAA GAC TCA GGC ATT GTA TTG AAG GAT GGG AAC CGG AAC CTC ACT ATC
Glu Thr Leu Val Glu Asp Ser Gly Ile Val Leu Lys Asp Gly Asn Arg Asn Leu Thr Ile
705

2341
CGC AGA GTG AGG AAG GAG GAC GAA GGC CTC TAC ACC TGC CAG GCA TGC AGT GTT CTT GGC
Arg Arg Val Arg Lys Glu Asp Glu Gly Leu Tyr Thr Cys Gln Ala Cys Ser Val Leu Gly
725

BamHI

2401
TGT GCA AAA GTG GAG GCA TTT TTC ATA ATA GAA GGT GCC CAG GAA AAG GGA TCC AGA TCT
Cys Ala Lys Val Glu Ala Phe Phe Ile Ile Glu Gly Ala Gln Glu Lys Gly Ser Arg Ser
745

2461
AAC TTG GGC TGG CTT TGT CTT CTT CTT TTG CCA ATT CCA CTA ATT GTT TGG GTG AAG AGA
Asn Leu Gly Trp Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
765

2521
AAC GAA GTA CAG AAA ACA TGC AGA AAG CAC AGA AAG GAA AAC CAA GGT TCT CAT GAA TCT
Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly Ser His Glu Ser
785

FIGURE 11C

```
2581
CCA ACC TTA AAT CCT GAA ACA GTG GCA ATA AAT TTA TCT GAT GTT GAC TTG AGT AAA TAT
Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp Val Asp Leu Ser Lys Tyr
                                                                          805

2641
ATC ACC ACT ATT GCT GGA GTC ATG ACA CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT
Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn
                                                                          825

2701
GGT GTC AAT GAA GCC AAA ATA GAT GAG ATC AAG AAT GAC AAT GTC CAA GAC ACA GCA GAA
Gly Val Asn Glu Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu
                                                                          845

2761
CAG AAA GTT CAA CTG CTT CGT AAT TGG CAT CAA CTT CAT GGA AAG AAA GAA GCG TAT GAC
Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala Tyr Asp
                                                                          865

                                                                  #
2821
ACA TTG ATT AAA GAT CTC AAA AAA GCC AAT CTT TGT ACT CTT GCA GGG AAA ATT CAG ACT
Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala Gly Lys Ile Gln Thr
                                                                          885

2881
ATC ATC CTC AAG GAC ATT ACT AGT GAC TCA GAA AAT TCA AAC TTC AGA AAT GAA ATC CAA
Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln
                                                                          905

2941
AGC TTG GTC TAG AGTGAAAAACAACAAATTCAGTTCTGAGTATATGCAATTAGTGTTTGAAAAGATTCT
Ser Leu Val ***
```

FIGURE 11D

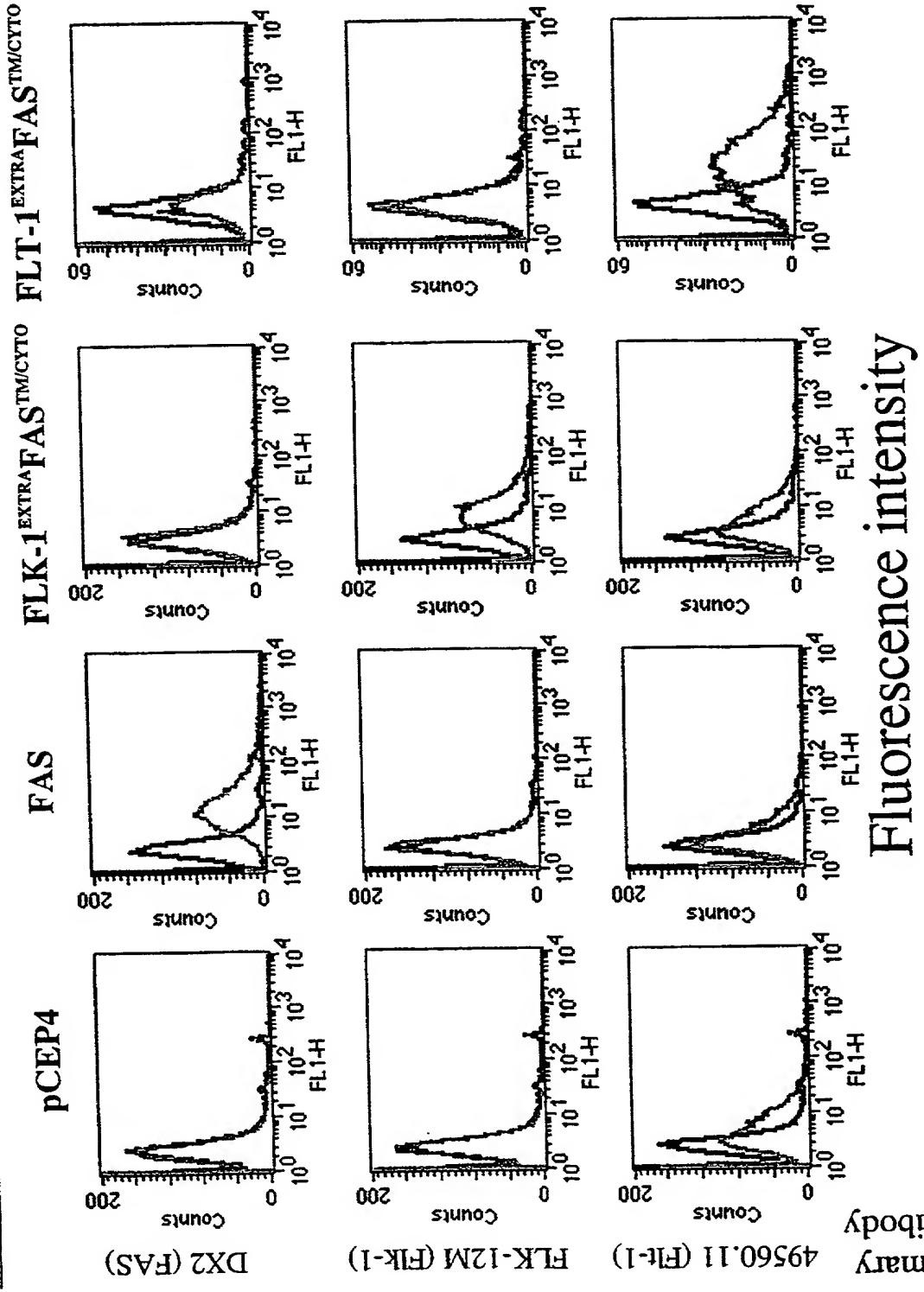
pCEP4
FAS
FLK-1EXTRAFAStm/cyto
FLT-1EXTRAFAStm/cyto
Primary antibody
4956O.11 (Flt-1)
FLK-12M (Flk-1)
DX2 (FAS)
Fluorescence intensity
Cell number
Counts
FL1-H

Figure 12

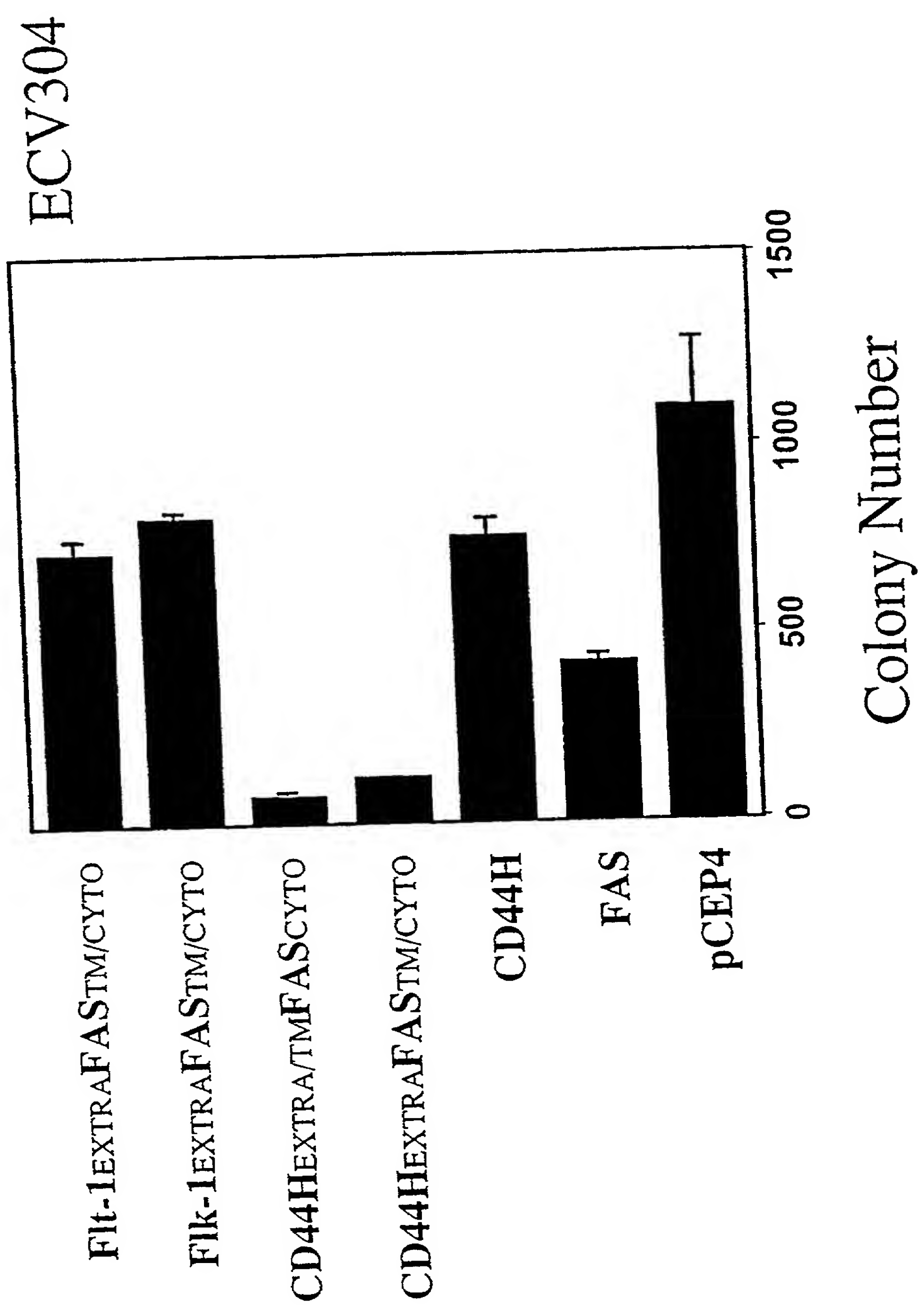
ECV304
Flt-1EXTRAFASTM/CYTO
Flk-1EXTRAFASTM/CYTO
CD44HEXTRA/TMFASCYTO
CD44HEXTRAFASTM/CYTO
CD44H
FAS
pCEP4
0
500
1000
1500
Colony Number

FIGURE 13

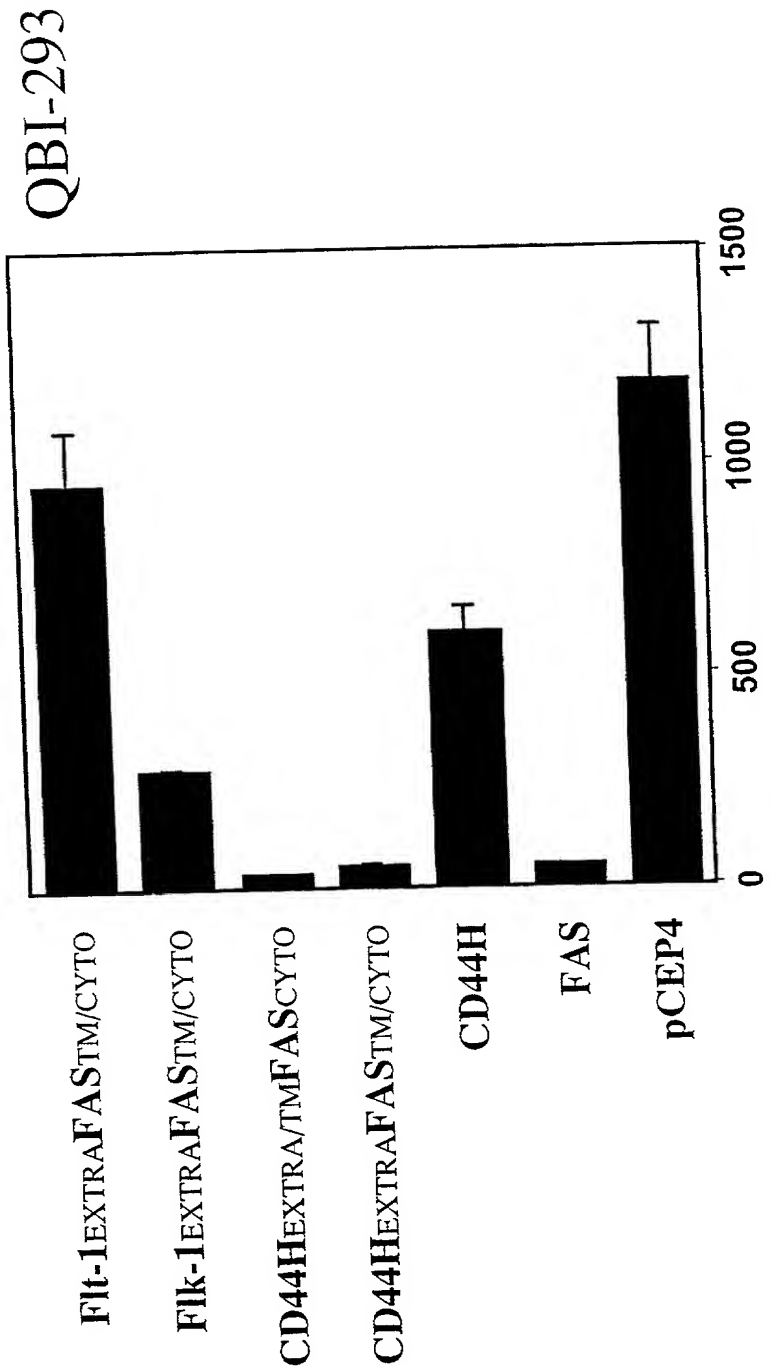

FIGURE 14

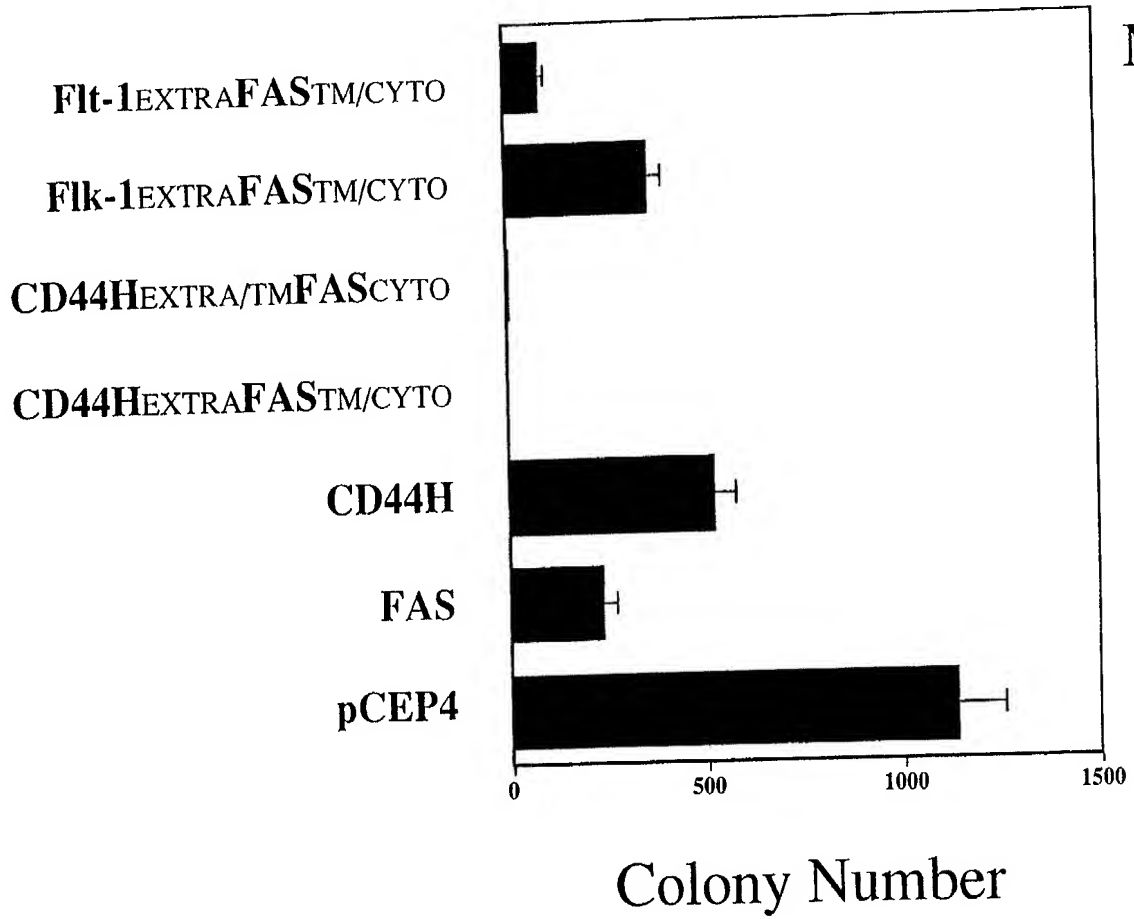
MCF-7
Flt-1EXTRAFASTM/CYTO
Flk-1EXTRAFASTM/CYTO
CD44HEXTRA/TMFASCYTO
CD44HEXTRAFASTM/CYTO
CD44H
FAS
pCEP4
0
500
1000
1500
Colony Number

FIGURE 15

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

the specification of which (check applicable box(s)):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____ (Atty Dkt. No. 620-179)
☒ was filed as PCT international application No. PCT/GB00/02449 on 26 June 2000
and (if applicable to U.S. or PCT application) was amended on 21 June 2001

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
9914650.8	Great Britain	24 June 1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below:

Prior U.S./PCT Application(s): Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned
PCT/GB00/02449	26 June 2000	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hosmer, 30184; Robert W. Faris, 31362; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097; Gary T. Tanigawa, 43180. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

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(Zip Code) OX9 5SX

2. Inventor's Signature: _____ Date: _____
Inventor: (first) (MI) (last) (citizenship)
Residence: (city) (state/country)
Mailing Address:
(Zip Code)

☐ *See attached sheet(s) for additional inventor(s) information!!*

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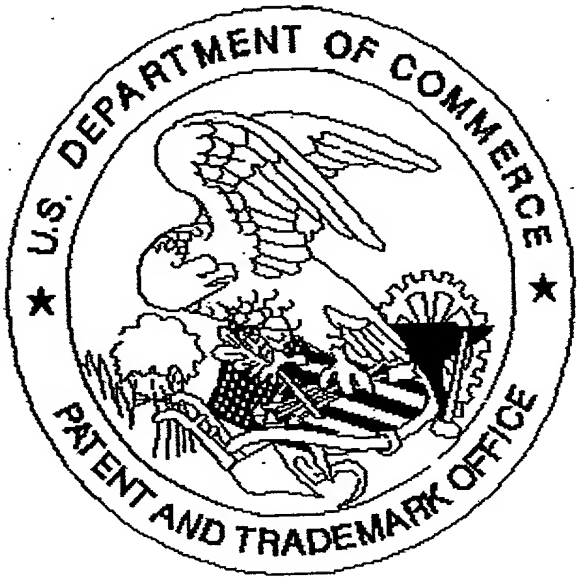

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